Generation of anti-(xanthine oxidoreductase) antibodies and their application to the quantification and purification of the mammalian enzyme

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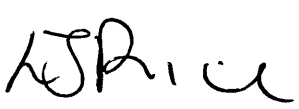

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Dedicated to the memory of my mother, June Price,

and to my father, Phil Price,

with much love

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Abstract

The enzyme xanthine oxidoreductase (XOR) has been implicated as a site of free radical generation in certain pathophysiological states, including chronic inflammation and ischaemia-reperfusion. However, investigations of its activity and location, particularly in human tissue, have been conflicting. There is, accordingly, a need for a means of detecting not only XOR enzymic activity, but also XOR protein, in tissue.

This thesis describes the generation of polyclonal and monoclonal (IgM) anti-(XOR) antibodies. An ELISA for XOR, with sensitivity in the nanogram range, is reported, and methods for affinity purification of XOR from tissue homogenates are described.

Abbreviations

ADP	Adenine diphosphate
AMP	Adenine monophosphate
ATP	Adenine triphosphate
CNBr-	Cyanogen-bromide-activated
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
Fab	Monovalent antigen-binding fragment of immunoglobulin molecule following papain digestion
FAD	Flavin adenine dinucleotide (oxidized)
$FADH_2$	Flavin adenine dinucleotide (reduced)
Fc	Crystallizable, non-antigen binding fragment of immunoglobulin molecule following papain digestion
Fe/S	Iron–sulphur redox centre
HRP	Horseradish peroxidase
HXO	Human xanthine oxidoreductase
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IgG	Immunoglobulin class gamma
IgM	Immunoglobulin class mu
I–R	Ischaemia–reperfusion
kDa	Kilodaltons
mAb	Monoclonal antibodies

mg	Milligram
Mo-P	Molybdo-pteridine redox centre
M_w	Molecular weight
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
ng	Nanogram
NRS	Normal rabbit serum
$O_2^{\cdot-}$	Superoxide anion radical
pAb	Polyclonal antibodies
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with 0.1% (v/v) Tween-20
PMSF	Phenylmethylsulphonyl fluoride
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

Contents

1 Introduction

1.1 Xanthine oxidoreductase

Xanthine oxidoreductase (XOR) is a dimeric metalloflavoprotein. It is a molybdenum iron-sulphur flavin hydroxylase. Members of this enzyme group catalyse reactions of the form:

$$RH \xrightarrow[+OH^-]{-2e,\ -H^+} ROH$$

where RH is the reducing substrate. The oxygen introduced into RH is from water and the electrons are accepted by the oxidizing substrate (Bray, 1975).

Xanthine oxidoreductase is the rate-limiting enzyme in nucleic acid degradation, catalysing the oxidation of hypoxanthine to xanthine, and xanthine to uric acid in the final stages of mammalian purine catabolism.

Most studies of XOR have used bovine enzyme (BXO) purified from milk (early studies reviewed in Bray, 1975; Massey *et al.*, 1969; Hart *et al.*, 1970; Nakamura and Yamazaki, 1982; Nishino *et al.*, 1982; Ventom *et al.*, 1988; Hunt and Massey, 1992), rat liver (Della Corte and Stirpe, 1968, 1972; Stirpe and Della Corte, 1969; Waud and Rajagopolan, 1976a, 1976b; Ikegami and Nishino, 1986; Saito *et al.*, 1989; Saito and Nishino, 1989; Amaya *et al.*, 1990; Ichikawa *et al.*, 1992) and chicken liver (Nishino and Nishino, 1989; Schopfer *et al.*,1988) because of the ready availability of source tissue.

More recently, studies have been initiated with human enzyme (HXO) purified from milk (Zikakis and McGinnis, 1988; Abadeh *et al.*, 1992) and liver (Krenitsky *et al.*, 1986; Xu *et al.*, 1994).

The enzyme monomer, molecular weight 150 kDa, has four redox centres:

- molybdo-pteridine
- two iron-sulphur clusters 2(Fe/S)
- flavin adenine dinucleotide (FAD).

These mediate electron transfer through the enzyme (Figure 1). Most reducing substrates interact at the molybdo-pteridine site and are hydroxylated as shown in the previous equation (Bray, 1975). NADH is not hydroxylated, but donates its electrons to the FAD in an oxidation reaction. Under physiological conditions, electrons are passed to oxygen or NAD^+ from the FAD centre. Some artificial electron acceptors, such as ferricyanide and methylene blue, can accept electrons from the Fe/S centres.

The predominant physiological form of XOR is xanthine dehydrogenase (XDH), which catalyses the oxidation of hypoxanthine or xanthine, with concomitant reduction of NAD^+ to NADH:

$$\text{Hypoxanthine} + H_2O + NAD^+ \rightarrow \text{Xanthine} + NADH + H^+$$

The oxidase form (XO) uses molecular oxygen as an electron acceptor, producing superoxide anion radical and/or hydrogen peroxide:

$$\text{Hypoxanthine} + 2O_2 + H_2O \rightarrow \text{Xanthine} + O_2^{\cdot-} + H_2O_2$$

$$\text{Xanthine} + 2O_2 + H_2O \rightarrow \text{Uric acid} + O_2^{\cdot-} + H_2O_2$$

Conversion of dehydrogenase to oxidase occurs reversibly by oxidation of reactive sulphydryl groups (14 in each 150 kDa subunit), and irreversibly by proteolysis (Figure 2; Stirpe and Della Corte, 1969, Della Corte and Stirpe, 1972).

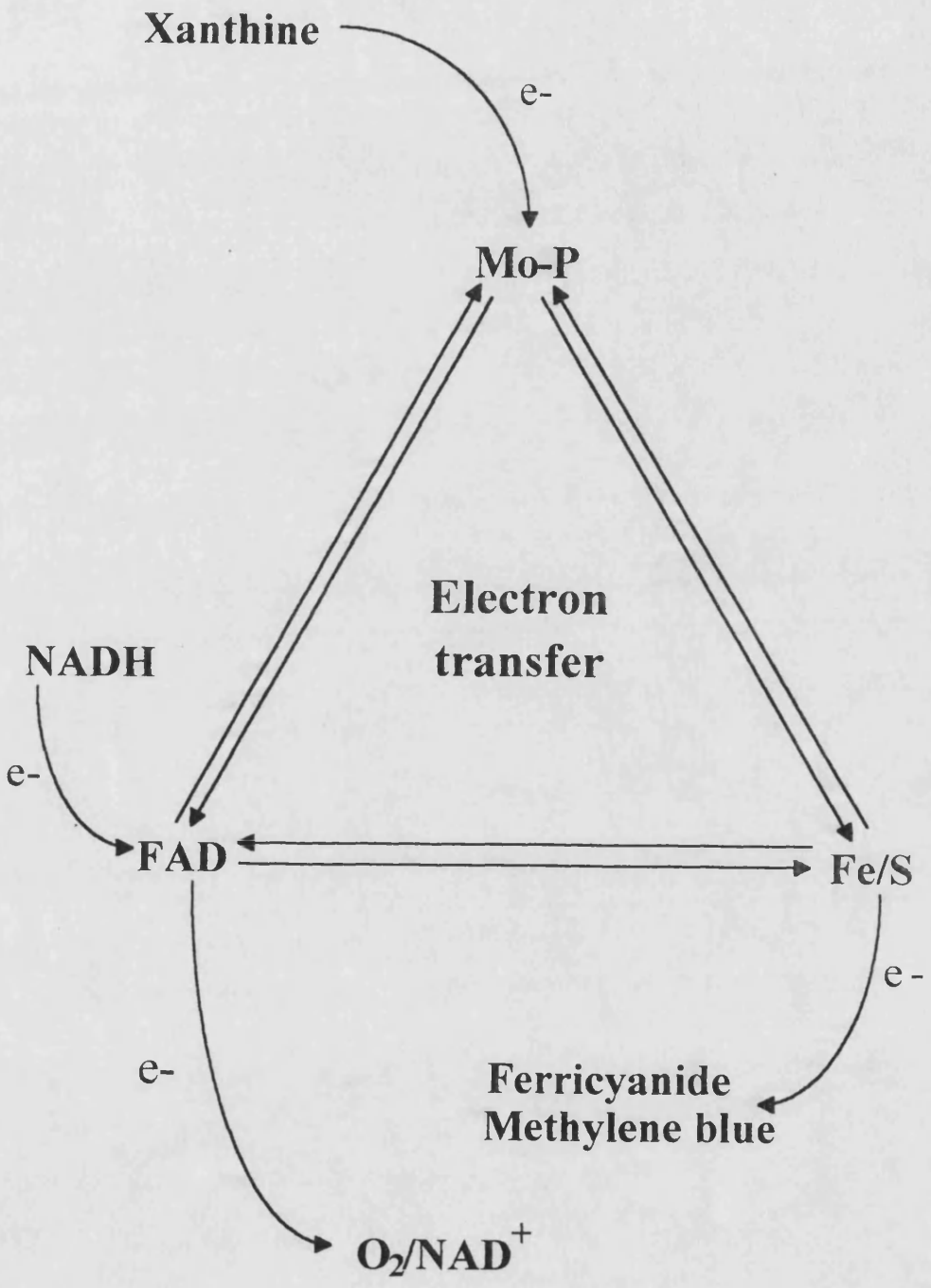


Figure 1. Redox centres in XO/XDH (modified from Edmondson *et al.*, 1973).

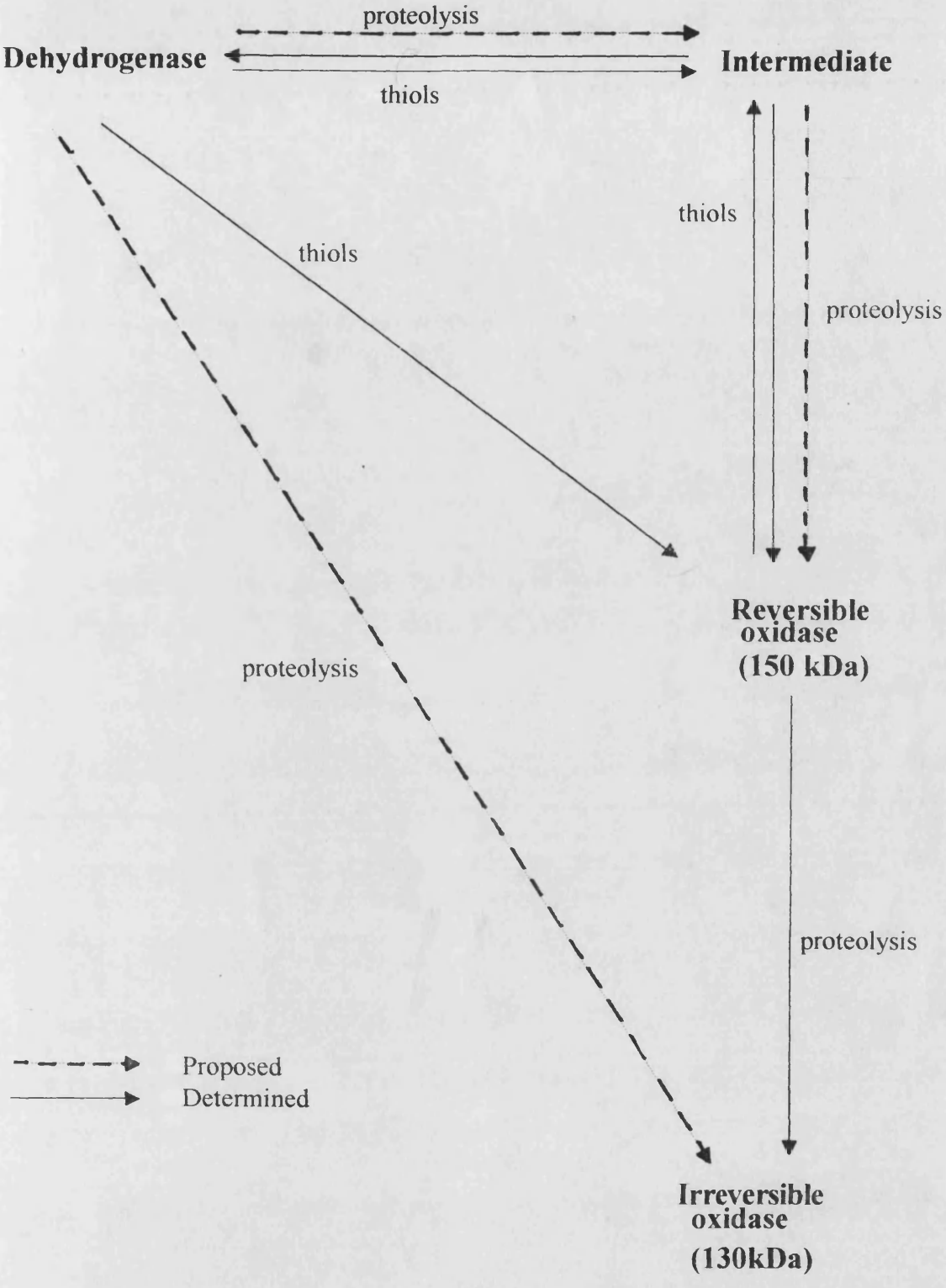


Figure 2. Interconversion of the various forms of XOR. From Parks and Granger, 1986 (modified from Della Corte and Stirpe, 1972).

The XOR enzyme can exist in forms in which one of the redox centres is dysfunctional – demolybdo, desulpho and deflavo (Bray, 1975).

Demolybdo-XOR lacks the molybdenum atom, but may retain some of the pteridine cofactor with which the molybdenum is normally associated (Ventom *et al.*, 1988). However, adding molybdate to demolybo-preparations only restores molybdenum cofactor activity to approximately 33% of 'normal' enzyme activity, indicating that the molybdenum site is not free in most of the demolybdo-molecules (Ventom *et al.*, 1988).

Demolybdo-XOR occurs naturally (Hart *et al.*, 1970; Bray, 1975; Abadeh *et al.*, 1992), and the ratio of demolybdo- to molybdo-containing-XOR in bovine milk appears to correlate with the nutritional status of the cow (Hart *et al.*, 1970; Ventom *et al.*, 1988). Demolybdo-XOR does not oxidize xanthine, but can react with NADH, which donates its electrons directly to FAD.

Desulpho-XOR contains a Mo=O group in place of the Mo=S group of the 'normal' enzyme (Bray, 1975). As the same redox centre is compromised as that in demolybdo-XOR, this form has characteristics similar to those of the demolybdo-enzyme. Although this form was originally to be an artifact of preparation or storage, there is growing evidence for its *in vivo* presence in bovine and human milk (Massey and Edmondson, 1970; Wahl and Rajapolan, 1982; Abadeh *et al.*, 1992), and speculation about its presence in other tissues (Abadeh *et al.*, 1992, 1993).

Deflavo-XOR, unlike desulpho- and demolybdo-XOR, is an artificial form. As it lacks the FAD cofactor, it cannot catalyse the oxidation of NADH. Deflavo-XOR can be prepared by:

- selective removal of the FAD cofactor under conditions of high ionic strength (Komai *et al.*, 1969)
- selective proteolysis with subtilisin at high pH (also removing part of the protein; Nagler and Vartanyan, 1976)
- alkylation of the FAD by treatment with iodoacetamide or phenylacetate (McGartoll *et al.*, 1970; Bray 1975).

Incubation of deflavo-XOR with flavin restores the native enzyme to approximately 85% of its original activity (Saito *et al.*, 1989; Komai *et al.*, 1969). This property has been exploited, by substituting ionizable or photoreactive FAD analogues, in order to study the FAD environment (Introduction 1.2.1; Nishino *et al.*, 1989; Saito *et al.*, 1989; 1992).

1.2 Distribution and characteristics of XOR

Xanthine oxidizing activity, similar to that found in eukaryotic XOR, has been detected in a variety of bacterial species - both Gram-negative and Gram-positive (Woolfolk and Downard, 1977). Activity has also been detected in invertebrates, but here the relative efficacy of the electron acceptor (i.e. NAD^+, ferricyanide or O_2) varies widely between species (Krenitsky *et al.*, 1974).

Birds, reptiles, amphibians and teleosts contain XOR with the following relative electron acceptor specificities: NAD^+ > ferricyanide > O_2 (Krenitsky *et al.*, 1974). In the same study, Krenitsky *et al.* (1974) reported the mammalian enzyme to have the electron acceptor specificity pattern: ferricyanide > O_2 > NAD^+, though they did not comment on the evolutionary significance of the patterns.

The emphasis of much of the work on XOR has concerned its distribution in mammalian tissues. As the focus of this project is on mammalian XOR, it is useful to summarize the results of previous studies on model mammalian species.

1.2.1 Rat

The presence of XOR in rat tissues has been described by many groups (early studies reviewed in Kooij A, 1994; Battelli *et al.*, 1972; Brunschede and Krooth, 1973; Krenitsky *et al.*, 1974; Kinuta *et al.*, 1989; Smith *et al*, 1989; Kooij *et al*, 1992a).

A comprehensive survey of rat tissues, using a specific histochemical method for XOR activity, indicated absence of enzyme in only the bladder,

cerebrum, cerebellum and aorta (Kooij *et al.*, 1992a). All other tissues showed positive staining, with high activity in epithelial and endothelial cells, and connective tissue.

Total XOR activity (i.e. XDH and XO) in liver is reported as 340 mU/g tissue, when 1 unit of enzyme activity corresponds to the formation of 1 μmole of product/minute (Battelli *et al.*, 1972). Total XOR activity of skeletal muscle (from hindquarters) is 11.0 mU/g wet weight of tissue (definition of activity as before; Smith *et al.*, 1989), while that of rat brain is 0.87 mU/g wet weight of tissue (Kinuta *et al.*, 1989).

Intracellularly, the enzyme has been located, using immunoelectron microscopy, in the cytoplasm of endothelial cells lining capillaries and small blood vessels in rat myocardium (Samra *et al.*, 1991). Cytosolic location of XOR has similarly been detected in rat hepatocytes (Ichikawa *et al.*, 1992).

Heart has been a focus of much attention because of the purported role of XOR in human ischaemia-reperfusion (I-R) injury (Introduction 1.3.1). Activities of the cardiac enzyme reported by different groups are summarized in Table 1.

Author	Total XOR activity (mU/g wet weight)	Assay (heart preparation)	Assay temperature
Schoutsen *et al.*, 1983	18	radiochemical HPLC (isolated, perfused)	30°C
Schoutsen *et al.*, 1983	10	radiochemical HPLC (homogenate)	30°C
Schoutsen *et al.*, 1983	27	radiochemical HPLC (homogenate, low M_w inhibitors removed)	30°C
Grum *et al.*, 1986	62	radiochemical (homogenate)	37°C
Kaminski *et al.*, 1986	28	spectrophotometric (homogenate)	25°C
Muxfeldt and Schaper, 1987	20	HPLC (homogenate)	Room temperature
Eddy *et al.*, 1987	47	spectrophotometric (homogenate)	Not reported
Eddy *et al.*, 1987	77	fluorimetric (homogenate)	Not reported
de Jong *et al.*, 1990	29	HPLC (isolated, perfused)	37°C

Table 1. Reported values of XOR activity in rat heart. One unit (U) of enzyme activity corresponds to the formation of 1 μmol of product/minute.

Kaminski *et al.*, (1986) reported that, with phenylmethylsulphonyl fluoride (PMSF) and dithiothreitol (DTT) included in the rat heart homogenization buffer, the dehydrogenase form accounted for 85.2% total XOR activity in rat heart, with oxidase accounting for 7.5% of activity. These workers also reported the presence of the intermediate form (7.2%), which preferentially transferred electrons to NAD^+ but, in the absence of this electron acceptor, could transfer electrons to oxygen. They found all three enzyme forms to be interconvertible on incubation with sulphydryl-modifying agents (in the absence of protease inhibitors). This indicates that the oxidase form in rat heart is the reversible, rather than the irreversible type.

The primary structure of rat liver XOR has been described (Amaya *et al.*, 1990). Freshly purified enzyme is dehydrogenase and comprises 1319 amino acid residues (M_w = 145 kDa). Trypsin digestion results in nicks in the sequence at residues 184–5 and 539–40 (Amaya *et al.*, 1990; Nishino and Tamura, 1991). Subsequent sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the fragments indicates the molecular weights of the three fragments produced, are 20 kDa, 40 kDa, and 85 kDa, corresponding to residues 1–184, 185–539 and 540–1319, respectively. That the three peptide fragments do not dissociate on trypsin digestion demonstrates a strong association between them.

The FAD cofactor and NAD^+ binding site are associated with the 40 kDa fragment; the 2 Fe/S sites are associated with the 20 kDa fragment and the molybdo-pteridine is associated with the 85 kDa fragment (Amaya *et al.*, 1990; Nishino and Tamura, 1991).

It has been suggested that the cysteine residues in the 20-kDa fragment contribute to the 2 Fe/S redox centres (Amaya *et al.*, 1990). These residues, and the surrounding amino acid sequences, are well conserved between the rat and *Drosophila melanogaster* enzymes (Amaya *et al.*, 1990). In total, the 20-kDa fragment of the rat enzyme has 66% homology with that of *D. melanogaster* (Amaya *et al.*, 1990). The 40-kDa fragment has less homology, with the amino acid sequence corresponding to the NAD^+ binding site differing between the XDH of *D. melanogaster* and rat. Identity between the 85 kDa fragments is 52% (Amaya *et al.*, 1990).

Nishino and Tamura (1991) report that, on treatment with fluorodinitrobenzene, a nucleophilic reagent, reversible conversion from XDH to XO occurs (Figure 3). This conversion corresponds to modification of the cysteine residues $Cys^{525, 980, 1313}$ and led the authors to speculate on the existence of a disulphide bridge between Cys^{525} and Cys^{980}.

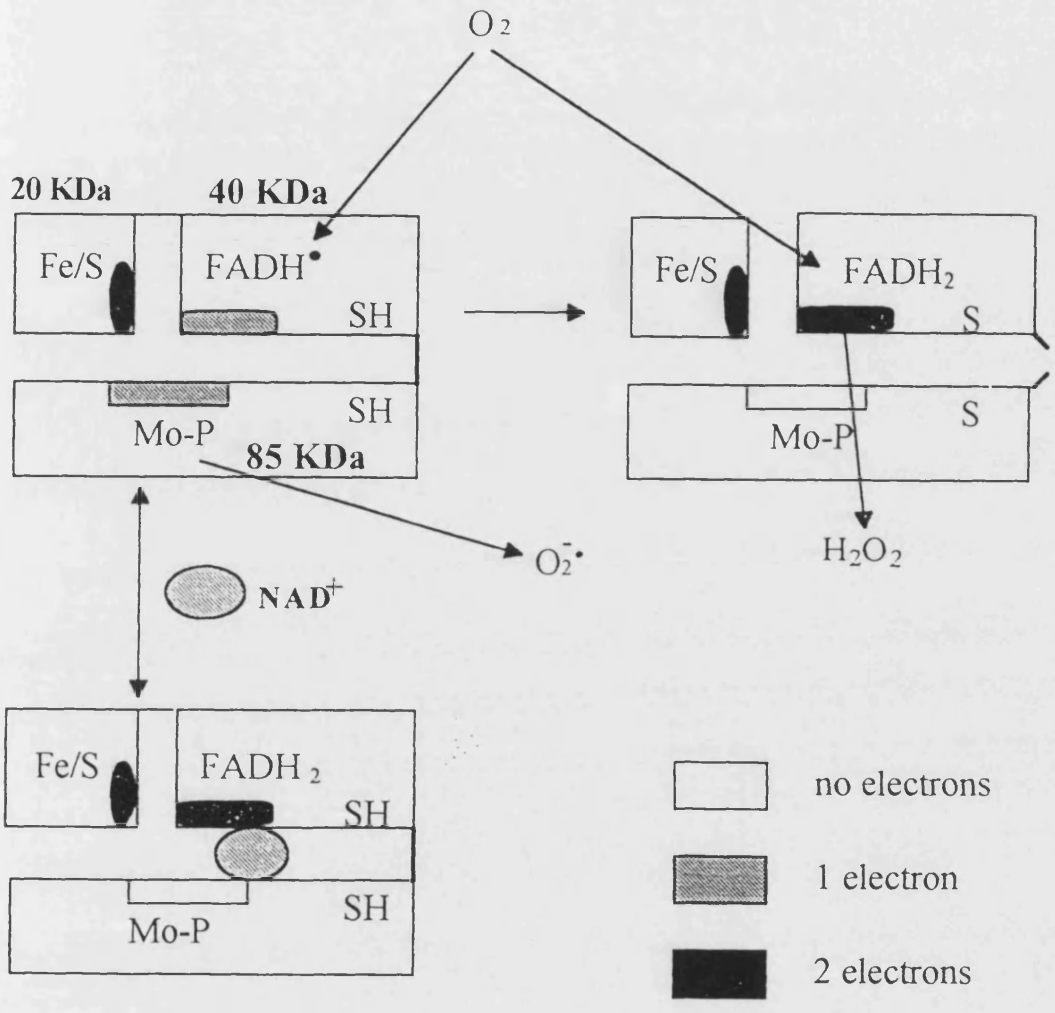


Figure 3. Conformational change by proteolysis, sulphydryl oxidation or NAD^+ binding. From Nishino and Tamura (1991).

Further evidence for the role of disulphide bridges in the dehydrogenase to oxidase conversion comes from studies using reconstituted XOR from deflavo-XOR and FAD. Holoenzyme reconstituted from non-DTT-reduced deflavo-XOR is wholly oxidase in nature, and has no activity towards NAD^+ (Saito *et al.*, 1989). On incubation with DTT, NAD^+-dependent activity reappears, with conversion of XO to XDH estimated as 60–70% (Saito *et al.*, 1989). Similarly, holoenzyme reconstituted from DTT-treated deflavo-XOR is largely dehydrogenase.

In the dehydrogenase enzyme, FAD is stabilized in the semiquinone state by flavin–protein interactions (Waud and Rajagopolan, 1976a; Nishino *et al.*, 1989; Saito and Nishino, 1989). Under standard assay conditions, the K_m for oxygen of the dehydrogenase enzyme is 260 μM, whereas that for the oxidase form is 46 μM (Saito and Nishino, 1989). This difference in affinity for oxygen is, most probably, due to the conformational changes around the FAD site. Conformational change could directly affect the reactivity of the FAD with molecular oxygen and/or alter the accessibility of the active site (Saito and Nishino, 1989).

On conformational modification, by proteolysis or sulphydryl oxidation, the stable form of FAD is fully reduced; $FADH_2$ reacts rapidly with oxygen to form hydrogen peroxide, rather than the superoxide anion radical formed by the semiquinone state (Nishino *et al.*, 1989; Saito and Nishino, 1989; Nishino and Tamura, 1991). The presence of NAD^+ also causes conformational change in the enzyme, again making fully reduced FAD the more stable state. This then rapidly reduces NAD^+ (Nishino and Nishino, 1989; Saito *et al.*, 1989).

1.2.2 Cow

Xanthine oxidoreductase has been located, using immunochemical procedures, in the capillary endothelial cells of bovine mammary gland, liver, heart, lung, intestine and in epithelial cells of lactating mammary gland (Jarasch *et al.*, 1981; Bruder *et al.*, 1982; Bruder *et al.*, 1983).

Bovine milk, particularly, contains high levels of BXO – around 50 mg/litre (Bray,1975) – and enzyme activity is much higher than that of other bovine tissues (Table 2).

Fraction	BXO activity (mU/mg protein)	Supernatant protein (%)
liver	1.4	0.1
milk (lipid globule preparation)	1.1×10^3	61
lactating mammary gland	2.8	1
non-lactating mammary gland	0.4	2.3
endocardium	0.13	0.01
brain	≤ 0.01	0.0005

Table 2. BXO in a range of tissues (from Bruder *et al.*, 1983). Activity was determined spectrophotometrically at 30°C; 1 unit (U) corresponds to the formation of 1 μmol urate/minute.

In milk, BXO has been localized to the lipid globule and lipid globule membrane, with the forms termed, respectively, 'soluble' and 'membrane bound' (Briley and Eisenthal, 1974; Bruder *et al.*, 1982). Briley and Eisenthal (1974) reported that the catalytic activities of the milk enzyme depended on its location, with the membrane-bound enzyme having enhanced NADH oxidation relative to that of xanthine. They used the ratio of xanthine oxidation to NADH oxidation (X/N) to distinguish between the two forms, and demonstrated that the X/N ratio decreased when free enzyme was incubated with a membrane preparation. Similar experiments indicated that only one type of binding site exists within the membrane, sites are independent, and that binding is reversible.

The enzyme found in endothelial cells appears similar to the milk form (Bruder *et al.*, 1982). As in milk, soluble and membrane-bound forms exist (Jarasch *et al.*, 1986). Although the enzyme purified from milk is usually 100% oxidase in nature, conversion to dehydrogenase can be achieved by incubation with DTT. The enzymic properties of BXO from milk are similar to those described for rat liver enzyme (Introduction 1.2.1).

Surprisingly, in view of the intensive study of BXO and the ready availability of bovine milk, enzyme from this source has only very recently been sequenced (Berglund *et al.*, 1996). Crystallization and X-ray diffraction studies are now underway (Eger *et al.*, 1994) and it is hoped that information on the three-dimensional structure will soon be available.

1.2.3 Rabbit

Xanthine oxidoreductase activity has been reported in rabbit liver, small intestine and kidney (Krenitsky *et al.*, 1974). As with other model species, attention has focused on rabbit heart because of the potential role of the enzyme in human ischemia-reperfusion injury (Table 3).

Author	Total XOR activity (mU/g wet weight)	Assay (heart preparation)
de Jong *et al.*, 1990	0.59	HPLC, 37°C (isolated, perfused)
Downey *et al.*, 1987	none	spectrophotometric, 37°C (homogenate)
Grum *et al.*, 1986	none	radiochemical, 37°C (homogenate)
Muxfeldt and Schaper, 1987	0.05	HPLC, room temperature (homogenate)
Schoutsen and de Jong, 1987	3×10^{-3}	radiochemical HPLC, 30°C (homogenate)
Terada, 1994	~ 98	HPLC, 37°C (butanol extracted homogenate)
Terada, 1994	~ 54	HPLC, 37°C (homogenate)
Wajner and Harkness, 1989	0.98 mU/mg protein (~ 21.6)	spectrophotometric, 25°C (homogenate)

Table 3. Reported values of XOR activity in rabbit heart; 1 unit (U) corresponds to the formation of 1 μmol urate/minute, except where indicated.

Rabbit heart, in comparison with reported values of XOR activity in rat heart (Table 1), has very low XOR activity, though reported values vary (Table 3). Reporting the highest activity, Wajner and Harkness (1989) used an assay mixture containing a high concentration of substrate. This led the authors to speculate on the presence, in the test sample, of a high-molecular-weight cytosolic enzyme inhibitor, the effects of which could be overcome at high substrate concentrations.

Terada (1994) reported XDH inhibition associated with an endogenous compound (M_w=370 kDa). By following the fate of NAD^+ in the presence of rabbit heart homogenate, Terada demonstrated that the suppression of dehydrogenase activity was associated with NADase activity, and speculated that it may be due to the action of a glycohydrolase. Incubation of the homogenate fraction with BXO (from milk) also suppressed dehydrogenase activity. The NADase activity was heat and acid labile, and was removed by butanol extraction.

1.2.4 Human

Xanthine oxidoreductase activity has been detected, using biochemical methods, in human liver, intestine, spleen, kidney and skeletal muscle (Watts *et al.*, 1965; Della Corte *et al.*, 1969; Krenitsky *et al.*, 1974; Mousson *et al.*, 1983). More recently, Kooij *et al.*, (1992b) have used a histochemical assay to demonstrate HXO activity in the liver and jejunum, but failed to detect activity in a range of other tissues.

Immunohistochemical techniques have localized the enzyme in the cytoplasm of endothelial cells lining the capillaries of heart kidney, brain, aorta, lung, mesentery, sinusoids, and alveolar epithelial cells of mammary gland (Jarasch *et al.*, 1986; Moriwaki *et al.*, 1993), and in vascular smooth muscle cells, macrophages and mast cells (Hellsten-Westing, 1993).

In addition, HXO located on the outer surface of the endothelial cell plasma membrane, has been reported (Adachi *et al.*, 1993). Its association with the plasma membrane is, reportedly, via heparin-like proteoglycans.

In human liver, XOR has specific activity comparable with that reported for bovine milk (1.8 U/mg HXO protein compared with 2.4–5.1 U/mg BXO protein; BXO data reviewed in Bray, 1975; Krenitsky *et al.*, 1986). Human XOR has been reported in milk at the level of 1.5–6 mg/100 ml (Abadeh *et al.*, 1992), and 6–14 mg/100 ml (Brown et al., 1995). Ninety-eight per cent of this HXO is inactive towards xanthine (Abadeh *et al.*, 1992); this is high in comparison with that reported (up to 60%) for BXO (Hart *et al.*, 1970; Bray, 1975). When NADH is used as the reducing substrate in assays of enzyme activity, however, the specific activities of both HXO and BXO from milk sources, are comparable (Table 4; Abadeh *et al.*, 1992). When activity is detected by measurement of urate or isoxanthopterin production, the assumption is that the enzyme has a functional molybdo-pteridine redox centre and, therefore, demolybdo and/or desulpho enzyme forms will not be detected by these assays. If the enzyme exists mostly in these forms in a given tissue, results from conventional assays of activity will be unreliable.

Assay substrate	Assay electron acceptor	HXO activity (mU/mg protein)	BXO activity (mU/mg protein)
xanthine	NAD^+	24	1060
xanthine	oxygen	15	524
xanthine	methylene blue	15	2440
xanthine	cytochrome c	106	1850
NADH	oxygen	39	31
NADH	methylene blue	149	244
NADH	cytochrome c	118	138

Table 4. Comparison of specific activities of HXO and BXO purified from milk (Abadeh et al., 1992); 1 unit (U) corresponds to the formation of 1 μmol urate/minute. Assays were performed at 22°C.

As with rabbit, reported values of HXO activity in human heart vary (Table 5). The presence and activity of HXO in human heart has become an issue of debate. It has been suggested that desulpho- and demolybdo-XOR, in similar proportions to those in human milk, may exist in the heart (Abadeh *et al.*, 1992); preliminary work in our laboratory supports this theory (Abadeh *et al*, 1993). Thus, in humans at least, varying amounts of different forms of XOR may exist, *in vivo*, in different tissues.

Interconversion between sulpho- and desulpho-XOR has been suggested as a means of molecular regulation of XOR activity (Itoh *et al.*, 1978; Furth-Walker and Amy, 1987; Brown *et al.*, 1995). By transferring sulphide ions, sulphur transferases could, for example, mediate regulation. Further studies are necessary to establish whether such interconversions can occur *in vivo* in human tissue.

Evidence for the interconversion between different forms of HXO was recently provided (Brown *et al.*, 1995). Activity and amount of HXO was measured in serial milk samples from women who had just given birth. Activity of the enzyme increased to a peak value and then decreased to basal level approximately 15 days post-parturition. Interestingly, corresponding reduction in HXO protein was not observed, suggesting activation-deactivation was occurring at the molecular level, possibly as a result of hormonal control.

Author	Total XOR activity (mU/g wet weight)	Assay and assay temperature (heart preparation)
Eddy *et al.*, 1987	0	spectrophotometric, fluorimetric, temperature not reported (homogenate)
Muxfeldt and Schaper, 1987	1.3	HPLC, room temperature (homogenate)
Grum *et al.*, 1989	0	radiochemical, 37°C (homogenate)
Wajner and Harkness, 1989	1.2 mU/mg protein (~37)	spectrophotometric, 25 °C (homogenate)
de Jong *et al.*, 1990	0.3	HPLC, 37 °C (isolated, perfused)
Podzuweit *et al.*, 1991	0	HPLC, 25°C (homogenate)
Kooij *et al.*, 1992b	negative	histochemical
Hellsten-Westing, 1993	positive (vascular smooth muscle cells, capillary endothelial cells, small venules and arterioles)	immunohistochemical
Moriwaki *et al.*, 1993	positive (endothelial cells)	immunohistochemical

Table 6. Reported activity and location of XOR in human heart; 1 unit (U) corresponds to the formation of 1 μmol urate/minute, except where indicated.

Recently, cDNA clones of human liver xanthine dehydrogenase have been isolated and sequenced, (Ichida *et al.*, 1993; Xu *et al.*, 1994). The 1333 amino acid sequence (Mw = 147 kDa) has 91% homology with rat XDH (Xu *et al.*, 1994). The trypsin cleavage sites are conserved, occurring between residues Lys^{185} and Asp^{186}, and at Lys^{552} in the human sequence (Ichida *et al.*, 1993; Xu *et al.*, 1994). The cysteine residues $Cys^{43, 48, 51, 73}$ and $Cys^{150, 169, 175}$ in the N-terminal region contributing to Fe/S centres I and II respectively, are also well conserved between rat and human (Xu *et al.*, 1994). The consensus NAD^+-binding site is located at residues 389–401 in the human sequence (Xu *et al.*, 1994). The sequence Gly-Xaa-Gly-Xaa-Xaa-Gly, contributing a βαβ helix formation in NAD-binding region of mouse and rat XDH has been described (Amaya *et al.*, 1990; Terao *et al.*, 1992), and is conserved in the human sequence at residues 42–47 and 796–801 (Xu *et al.*, 1994). The molybdenum binding site is also well conserved between rat and human, occurring in the 85-kDa fragment (Amaya *et al.*, 1990; Xu *et al.*, 1994).

Using Northern blot analysis, the tissue expression of the XDH gene has been studied (Ichida *et al.*, 1993; Xu *et al.*, 1994). The gene, which is located on chromosome 2 (Ichida *et al.*, 1993), is expressed in all tissues including the heart, brain, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and also in the placenta (Xu *et al.*, 1994).

1.3 Role of XOR as a source of reactive oxygen species

Free radicals are molecules, or molecular fragments, containing unpaired electrons in the outer orbit; they can act as an oxidants or reductants. These chemically reactive species can initiate chain reactions which can result in irreversible structural damage to lipid and protein.

Reported sources of free radicals, in addition to the XOR system, include activated neutrophils (McCord 1987), the reduced mitochondrial electron transport chain (donating electrons directly to molecular oxygen), catecholamine oxidation, prostaglandins and drugs such as doxorubicin (Kukreja and Hess 1992). Where the balance between free radical generation and neutralization is disrupted, tissue damage may occur. Free radical-mediated tissue damage has been implicated in I–R injury (McCord, 1985) and has been cited as contributing to the pathobiology of a number of diseases including myocardial infarction, rheumatoid arthritis, pancreatitis and atherosclerosis (reviewed in Kooij, 1994).

1.3.1 A hypothesis of XO-mediated damage during I–R (McCord, 1985)

During ischaemia, the ATP level in a cell diminishes and the AMP concentration increases concomitantly. The AMP is then catabolized to adenosine, inosine and, finally, hypoxanthine – a substrate of XOR. The drop in ATP concentration also results in disturbance of the ionic gradients across the cell membranes which, in turn, causes a redistribution of calcium ions within the cell. High cytosolic concentrations of calcium ions activate a protease that converts the

dehydrogenase enzyme form to the oxidase form.

On restoration of blood flow, molecular oxygen becomes available to the oxidase form enabling it to oxidize hypoxanthine, producing superoxide anion and/or hydroxyl radicals (Figure 4).

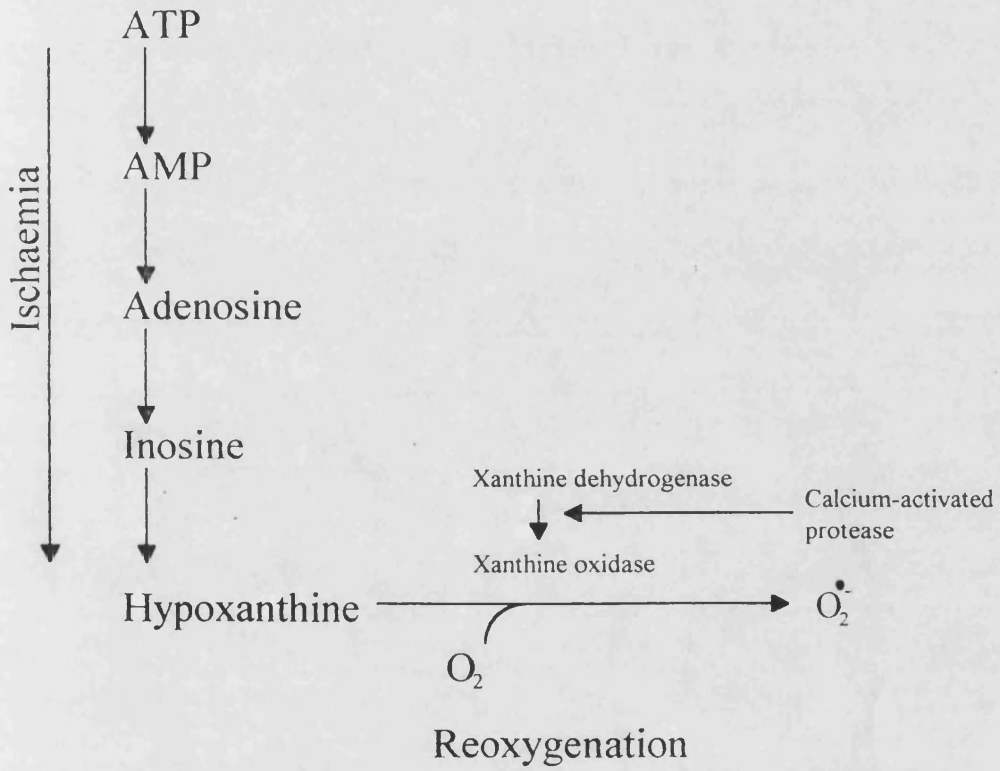


Figure 4. A hypothesis of XO-mediated damage during I–R (McCord, 1985).

1.3.2 Inhibition studies

The major evidence for the involvement of XO-mediated free radical damage in I-R comes from studies using the XOR inhibitor, allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine). Allopurinol is an alternative-substrate inhibitor, competing with hypoxanthine and xanthine for the substrate binding site; XOR oxidizes allopurinol to oxypurinol (4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine), which is a non-competitive inhibitor. Reports of allopurinol and oxypurinol acting as direct free radical scavengers do, however, require that reports of beneficial effects on infarct size should be rigorously assesed before firm conclusions from allopurinol inhibition studies can be drawn (Moorhouse *et al.*, 1987; Hoey *et al.*, 1988; Grootveld *et al.*, 1988).

Chronic administration of allopurinol (i.e. 18-24 hours prior to coronary artery occlusion) has been reported to limit infarct size in the canine model of I-R (Akizuki *et al.*, 1985; Chambers *et al.*, 1985; Werns *et al.*, 1986). The nature of the inhibitory effect exerted by allopurinol has been questioned (Werns *et al.*, 1991). When allopurinol was administered to dogs shortly (i.e. 30 minutes) before coronary artery occlusion, no effect on infarct size was observed (Reimer and Jennings, 1985). In contrast, short-term administration of oxypurinol has been reported to limit infarct size following I-R (Werns *et al.*, 1991). However, the same authors demonstrated that this was not due to XOR inhibition as amflutizole, an XOR-inhibitor chemically unrelated to allopurinol, failed to limit infarct size under the same conditions (Werns *et al.*, 1991; Werns and Lucchesi, 1990). Amflutizole inhibition of XOR in rat heart has, however, been reported to protect

against reperfusion-induced arrhythmias associated with free radical production (Manning *et al.*, 1988).

In species where the existence of XOR in heart is controversial, conflicting theories have arisen as a result of inhibition studies. Some groups report that allopurinol does not limit infarct size in the rabbit (Yellon *et al.*, 1985; Downey *et al.*, 1987), while Terada *et al.*, (1991) report that allopurinol does have protective effects. Using explanted human hearts perfused with hypoxanthine, de Jong *et al.*, (1991), demonstrated low HXO activity, concluding that HXO-mediated cardiac damage was unlikely to occur. Johnson *et al.*, (1991), however, described a beneficial effect of allopurinol administered to patients undergoing bypass surgery.

Human umbilical vein endothelial cells (HUVEC) have been proposed as a good *in vitro* model for endothelial cells *in vivo* (Michiels *et al.*, 1992). Using these cells, parameters of cell viability and free radical production were reported under different conditions; allopurinol was reported to completely inhibit superoxide production (Michiels *et al.*, 1992). The authors also report a measurable XO activity following, but not preceding, ischaemia.

1.3.3 NADH oxidase activity of XOR

The major assumption of the above model of XOR-mediated free radical damage following I–R is that the conversion of XDH to XO occurs rapidly enough to account for the free radical production reported. The rate of XDH to XO conversion in ischaemic rat intestine is, reportedly, rapid with complete conversion occurring in less than 1 minute (Roy and McCord, 1983). The speed and efficiency of the conversion have, however, been questioned as Nishino and

Tamura (1991), could only demonstrate conversion in post-I-R dog lung of around 30% at maximum.

The NADH oxidase activity of XOR also results in superoxide generation (Nishino and Tamura, 1991; Abadeh *et al.*, 1992), and a case can be made for free radical generation via NADH oxidase activity during post-ischaemia reperfusion.

- Physiological NADH levels increase during ischaemia and the NAD^+/NADH ratio decreases from 700 to 30 (Nishino and Tamura 1991).
- Xanthine dehydrogenase is more efficient than XO in oxidizing NADH (Powell, 1996), and XDH to XO conversion is not required.
- HXO is reported as having low activity when assayed using conventional measurement of urate production, but has NADH activity comparable with that of BXO (Table 4; Abadeh *et al.*, 1992).
- NADH oxidase activity is not inhibited by conventional XOR inhibitors (Powell, 1996), and evidence based on allopurinol, oxypurinol or amflutizole inhibition is irrelevant to a pathogenic mechanism based on NADH oxidation.
- Naturally occurring desulpho enzyme can oxidize NADH but not hypoxanthine or xanthine. *In vivo* mechanisms of conversion between holoenzyme and desulpho XOR have yet to be determined.

Thus, while the 20-30% of XOR existing as, or converted to, XO *in vivo* may follow the McCord model, NADH oxidation, which does not require XDH→XO conversion may also contribute. The balance between these two activities may explain some of the incongruous results of various inhibition studies.

1.3.4 Inflammation - a reported role for XOR

During the acute inflammatory process, activated neutrophils permeate the vascular wall and adhere to endothelial cells at and around the site of inflammation. The stimuli that attract the neutrophils to the site of inflammation also induce changes membrane that promote fusion of the neutrophil granule membrane, and subsequent exocytosis of the granules' contents.

Incubation of stimulated neutrophils with extracts of rat pulmonary artery endothelial cells has been shown to increase XO activity without increasing the total XOR activity (Phan *et al.*, 1989). Further experiments have implicated neutrophil elastase as the mediator of this conversion, as incubation of whole endothelial cells with purified neutrophil elastase caused a similar increase in XO activity (Phan *et al.*, 1992). Elastase is present in neutrophil granules and, on exocytosis, pericellular levels of elastase increase. As a result of the neutrophil/endothelial cell association, it has been suggested that neutrophil elastase enters the endothelial cell and causes the conversion of XDH to XO (Phan *et al.*, 1992).

In opposition to the McCord theory of XO-mediated cellular damage during I-R, Phan *et al.* (1994), demonstrated that the action of intracellular calcium proteases was not sufficient to cause the observed XDH to XO conversion. Activated neutrophils generate reactive oxygen species via NADPH oxidase located on the extracellular side of the plasma membrane. The superoxide anion radical produced by NADPH oxidase dismutates to hydrogen peroxide, which may then diffuse into the endothelial cell (Varani *et al.*, 1990).

$$O_2^{-}\bullet + 2H^+ \xrightarrow{+1e^-} H_2O_2$$

An effect of hydrogen peroxide within the cell is to diminish cellular ATP reserves, with a consequent increase in the concentration of XOR substrates, xanthine and hypoxanthine (Varani *et al.*, 1990). Xanthine oxidase-generated superoxide anion radicals can react with ferric iron to produce ferrous iron which can then react with NADPH oxidase-generated hydrogen peroxide to produce ferric iron and the highly reactive hydroxyl radical.

The whole reaction sequence is termed the Haber-Weiss reaction.

$O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2$

$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$

$H_2O_2 + O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO\cdot$

Intracellular free radicals can react with plasma membrane lipids to produce the chemotactic agents that attract neutrophils to the site of inflammation (Perez *et al.*, 1980). Thus, XO may amplify the inflammatory response by recruiting increasing numbers of neutrophils to the locale.

A similar hypothesis links free radical-stimulated neutrophil recruitment to I-R injury (Granger, 1988; Korthuis and Granger, 1993). Oxidants generated by XO on reperfusion, initiate and/or upregulate pro-inflammatory agents which attract and activate neutrophils. As described above, the combination of NADPH oxidase and XO activities can result in the generation of the highly destructive hydroxyl radical.

The oxidative effects of free radicals within a pathophysiological environment are likely to be highly damaging and wide-ranging, as illustrated by the scenario proposed for oxidative damage in inflammatory joint disease (Figure 5; Winrow *et al.*, 1993).

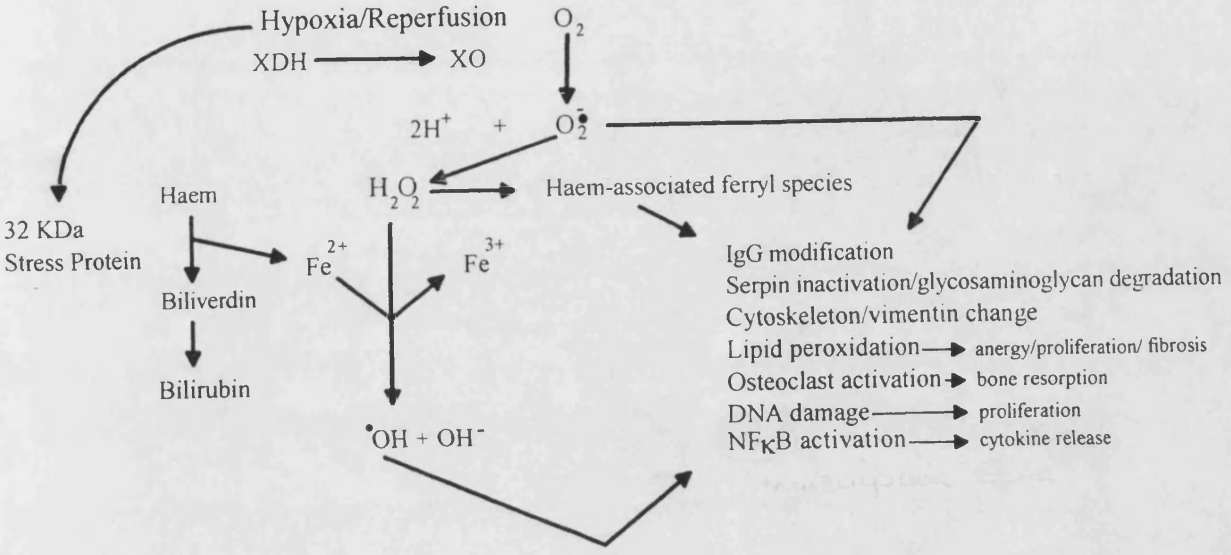


Figure 5. Proposed mechanisms of free-radical-mediated damage in inflammatory joint disease (Winrow *et al.*, 1993).

1.3.5 The effect of cytokines and a glucocorticoid on XOR activity

In addition to direct XDH to XO conversion, XOR activation by inflammatory mediators has also been reported (Dupont *et al.*, 1992; Pfeffer *et al.*, 1994). The effect of cytokines and steroids on XOR activity has been studied using cultured rat lung endothelial cells and bovine renal epithelial cells (Dupont *et al.*, 1992; Pfeffer *et al.*, 1994) as model systems.

Dupont *et al.* (1992) reported that, though cultured rat lung microvascular cells have low levels of XOR activity (approximately 2.0 mU/g protein), 24-hour exposure to 1000 U/ml interferon-γ (IFN-γ) increased activity fivefold. Xanthine oxidase activity contributed approximately 20% of the activity before stimulation, and this ratio was maintained following IFN-γ stimulation. Northern blot analysis showed increased levels of XOR mRNA on IFN-γ stimulation, indicating upregulation of mRNA transcription. Actinomycin D and cycloheximide abolished the effect of IFN-γ on XOR activity, providing further evidence that transcription of the XOR gene increases as a result of IFN-γ stimulation. Dupont *et al.* (1992) also demonstrated that cycloheximide, alone and in combination with IFN-γ, increased XOR mRNA levels. This result indicates that a constitutively produced repressor protein, or protein with RNase activity, may regulate XOR expression.

Similar results have been reported for bovine renal epithelial cells (Pfeffer *et al.*, 1994). Again, 1000 U/ml IFN-γ increased XOR activity from approximately 40 mU/g protein to 57 mU/g protein, with XO accounting for 40–50% total activity both before and after stimulation. Similarly, cycloheximide was shown to increase the level of XOR mRNA. Unlike Dupont *et al.* (1992), however, Pfeffer *et al.* (1994) also found that exposure to TNF, IL-1 and IL-6 also increased XOR activity in cells by transcriptional upregulation. Furthermore, it appeared that these cytokines had an additive effect on XOR activity, though the mechanisms by which this occurred were not investigated.

Dexamethasone, a glucocorticoid, also stimulates XOR transcript[illegible] expression and activity, acting apparently via a glucocorticoid receptor (Pfeffer *et al.*, 1994). This result complicates the hypothesis that XOR-generated free radicals contribute to tissue damage during inflammation, as dexamethasone is an anti-inflammatory agent.

The reported effects of cytokines and a glucocorticoid on XOR activity, together with the actions of proteases and the postulated actions of sulphydryl modifiers, suggest that XOR activity may be controlled at two levels:

- transcriptional level – upregulation via cytokines and glucocorticoids, and the action of a repressor protein
- tertiary level – conformational modification resulting in XDH to XO conversion.

1.4 Aims of the project

Xanthine oxidoreductase is constitutively expressed in a wide range of cells. Its 'dual nature' and properties have interested researchers for many decades, and its involvement in different diseases and pathological responses has been postulated.

Research into the conversion of the dehydrogenase form to the oxidase form, transcriptional activation and regulation via conversion from inactive to active forms, has made the study of XOR both complex and exciting. We still do not know which is the most important role of the enzyme. It could be production of the antioxidant, uric acid, or it could involve its ability to produce free radicals under certain conditions. Knowledge of XOR regulation will contribute to our understanding of why the enzyme exists.

In order to establish the level of regulation and activation of XOR, we must follow both activity and levels. The aim of the project was to generate monoclonal and polyclonal anti-(XOR) antibodies (mAbs), recognising different enzyme epitopes which could be used, in ELISA, to quantitate the enzyme in mammalian tissues and cell culture systems. ELISA was chosen as the assay of choice as this method is convenient, safe, reproducible and relatively inexpensive. Quantitative data would complement kinetic assays of enzyme activity in experiments designed to address the mechanisms of regulation of XOR. The anti-(XOR) antibodies were also required for affinity purification of XOR from tissue, and methods for each purification were to be investigated. Again, immunoaffinity chromatography is a powerful yet simple method for isolating proteins.

2 Materials and methods

2.1 Preparation of standard solutions

All chemicals were purchased from Sigma Chemical Co. Ltd, unless otherwise stated. Addresses of suppliers are given in Appendix I.

All dilutions in distilled water unless otherwise stated.

Phosphate buffered saline (PBS)

sodium chloride	0.12 M
disodium hydrogen phosphate (anhydrous)	0.024 M
potassium dihydrogen phosphate	0.006 M

Phosphate-buffered saline-Tween (PBS-T)

as PBS with 0.1% (v/v) Tween-20

2.2 Standard procedures

2.2.1 Protein estimation - Lowry method (modified from Lowry *et al.*, 1951)

A series of standard protein solution dilutions from 0–200 μg/200 μl was prepared, from a 1 mg/ml stock, in the same buffer as the test sample. The reference protein was either bovine serum albumin or a standard concentration of BXO. A volume of cupric sulphate 1% (w/v) was added to an equal volume of sodium potassium tartrate 2% (w/v). This was mixed 1:50 with sodium carbonate 2% (w/v) in 0.1 M sodium hydroxide prior to the assay. Of this solution, 1 ml was added to the 200 μl protein standard or test sample. After incubation for 10 minutes at room temperature, 100 μl Folin and Ciocalteu's Phenol reagent [50% (v/v); BDH Chemicals Ltd] was added. The tubes were vortex mixed and incubated for 30 minutes. Samples (100 μl) were transferred to a 96-well plate and the colour reaction was quantitated by a Dynatech Mini-skan II EIA plate reader (Labsystems) at 690 nm. The unknown protein concentration could be determined from a reference plot of absorbance at 690 nm against protein concentration for the standard solution.

Precipitation occurred with some buffers and in these cases, the supernatant was used where possible. A sample reference curve is shown (Figure 6) with average values from two assays using different stock solutions and performed on different days. The working range of the assay was 40–140 μg protein.

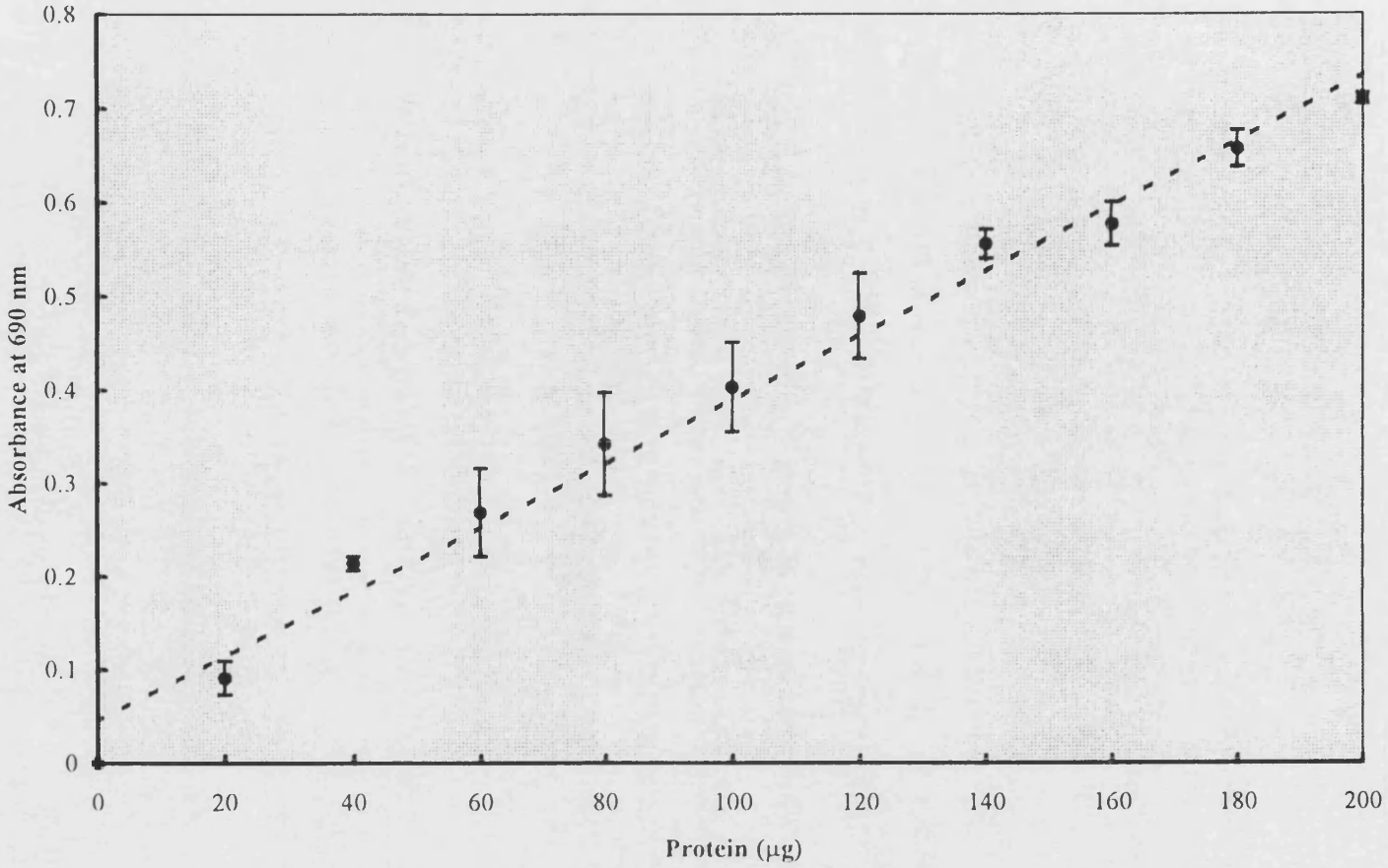


Figure 6. Protein content estimation using a method modified from Lowry *et al.*, (1951).

The points represent the means of duplicate assays, and the vertical bars indicate the range of values.

2.2.2 Protein estimation - Bradford method (modified from Bradford, 1976)

When interference occurred using the Lowry method of protein estimation, the Bradford dye-binding assay was substituted.

Bovine serum albumin or BXO was used as the standard protein solution. The standard was diluted over a range 0–100 μg in a sample volume of 100 μl. The volume of test sample was adjusted to a final volume 100 μl. Of the Bio-Rad dye [20% (v/v); Bio-Rad Laboratories Ltd], 1 ml was added to each sample and the mixture was vortex mixed. After 15 minutes, 100 μl samples were transferred to a 96-well plate and the A_{595} was measured using the Dynatech plate reader. The protein concentration in the test sample was estimated from a reference plot of A_{595} against protein concentration of the standard dilutions.

When test sample volumes were small, the assay was scaled down by a factor of 10 and performed in a 96-well plate.

Again, a sample reference curve is shown (Figure 7) with average values from two assays using different stock solutions and performed on different days. The working range of the assay was 0–100 μg protein.

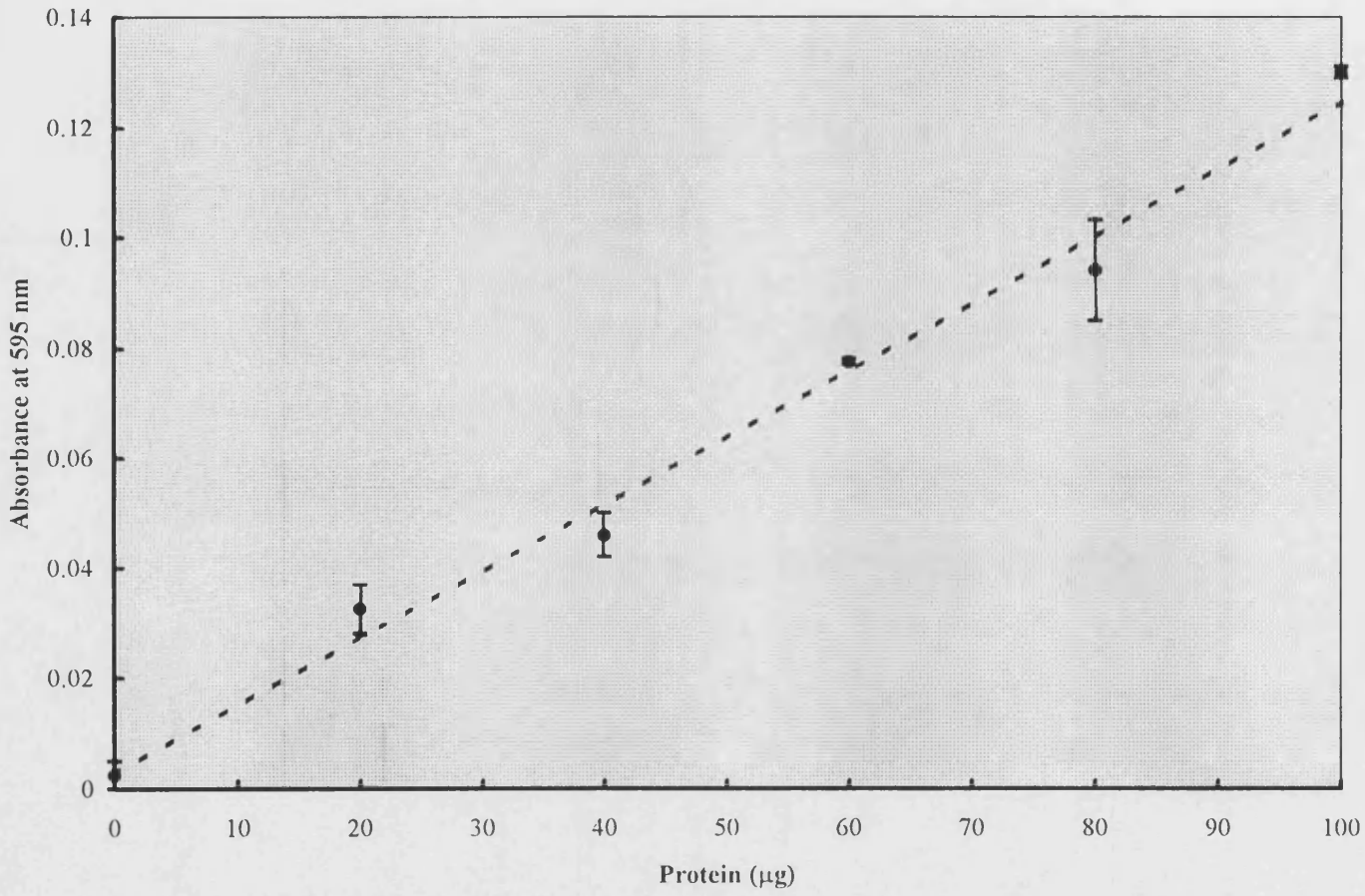


Figure 7. Protein content estimation using a method modified from Bradford (1976).

The points represent the means of duplicate assays, and the vertical bars indicate the range of values.

2.2.3 SDS-PAGE (modified from Laemmli, 1970)

Vertical gels, 10% separating gel and 5% stacking layer, were prepared routinely. The separating gel comprised 10% (w/v) acrylamide (Protogel 37.5:1 acrylamide to methylene bisacrylamide solution; National Diagnostics), 0.375 M Tris-HCl, pH 8.8, 1% (w/v) SDS, 0.06% (v/v) TEMED, 0.03375% (w/v) ammonium persulphate. The stacking layer comprised 5% (w/v) acrylamide (Protogel), 0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) TEMED, 0.0525% (w/v) ammonium persulphate.

Samples containing approximately 50 μg protein were dialysed against a weak buffer when necessary, and incubated at 100°C with an appropriate volume of sample buffer [0.062 M Tris-HCl, pH 6.8, 1% (w/v) SDS, 50% (v/v) glycerol, 10% (v/v) mercaptoethanol, 0.005% (w/v) bromophenol blue] for 1 minute.

Particles or precipitates in the sample were pelleted by centrifugation and the supernatant was loaded into a well in the stacking layer.

At least one lane contained unstained molecular weight markers (SDS-6H) in order to calibrate the gel:

Carbonic anhydrase (bovine erythrocytes)	29 kDa
Ovalbumin	45 kDa
Albumin (bovine plasma)	66 kDa
Phosphorylase B (rabbit muscle)	97.4 kDa
β-Galactosidase (*E. coli*)	116 kDa
Myosin (rabbit muscle)	205 kDa

Samples were electrophoresed at 60 V for approximately 30 minutes or until the protein had entered the gel. The voltage was then increased to 200 V for approximately 5 hours. The electrophoresis buffer contained 0.025 M Tris-HCl, pH 8.3, 0.192 M glycine and 0.1% (w/v) SDS.

The gels were fixed and stained in 45% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R, and were subsequently destained in solution containing 5% (v/v) methanol and 7.5% (v/v) acetic acid.

Molecular weight estimation was made by comparison of the R_f value of the sample against a calibration curve of R_f against molecular weights of the molecular weight markers.

2.2.4 Western blot

In order to probe discrete electrophoretic bands with specific antibodies, proteins were transferred to nitrocellulose. The nitrocellulose (Bio-Trace NT; Gelman Sciences Ltd) was soaked in blot buffer, pH 8.3, comprising 0.025 M Tris, 0.192 M glycine, methanol 20% (v/v), before sandwiching with the SDS-PAGE gel and several thicknesses of absorbent paper. This was then placed in the blot tank (Bio-Rad Laboratories Ltd) containing blot buffer. Proteins were transferred with a constant voltage 30 V for 16 hours at 4°C.

The nitrocellulose was washed with three changes of PBS-T; free binding sites were blocked by incubation with PBS-T containing 1% (w/v) casein for a minimum of 1 hour at room temperature.

The PBS-T/casein was discarded and the nitrocellulose was washed as before. The blot was incubated with the test antibody, diluted in PBS-T/casein,

for 2–3 hours. The antibody solution was discarded and the nitrocellulose was washed. It was incubated with an appropriate volume of species- and isotype-specific, biotinylated antibody 0.1% (v/v) in PBS-T/casein (Vectastain ABC Kit, Vector Laboratories Ltd) for a minimum of 1 hour.

This antibody solution was discarded and the nitrocellulose was washed thoroughly. The blot was incubated for a minimum of 1 hour with PBS-T/casein containing pre-formed biotin-avidin HRP complexes 0.5% (v/v) (Vectastain ABC Kit).

The blot was washed with PBS-T and further washed with PBS. It was incubated in horseradish peroxidase (HRP) substrate, 0.5% (v/v) 4-chloro-1-naphthol [from stock 3% (w/v) in methanol] and 0.012% (v/v) hydrogen peroxide, in PBS, overnight at room temperature. The reaction was stopped by washing with PBS.

2.2.5 Spectrophotometric assay for total XOR activity (Avis *et al.*, 1956)

The rate of urate production in aerated 0.05 M potassium phosphate, pH 8.3, with 10^{-4} M xanthine and $5x10^{-4}$ M NAD^+ was followed spectrophotometrically at 295 nm.

The extinction coefficient for uric acid is 9.6×10^3 $M^{-1}cm^{-1}$.

2.2.6 Fluorescent assay for XOR activity (Beckman *et al.*, 1989)

The sensitivity of this assay is two orders of magnitude greater than the spectrophotometric measurement of urate production. Pterin interacts with the enzyme at the same active site as xanthine. The enzyme catalyses the conversion of pterin to isoxanthopterin. Methylene blue acts as an electron acceptor allowing measurement of dehydrogenase activity.

Fluorescent measurement was made by a Perkin Elmer LS-5B fluorimeter with $\lambda_{excitation}$ 345 nm and $\lambda_{emission}$ 390 nm and slit width 5 nm. The assay buffer was 0.05 M potassium phosphate with 10^{-5} M pterin for measurement of XOR activity. Methylene blue, 10^{-5} M, was added to the assay mixture to allow measurement of dehydrogenase activity. Xanthine oxidoreductase activity was inhibited with allopurinol $4x10^{-5}$ M. The assay was calibrated by addition of isoxanthopterin standards.

2.2.7 Screening ELISA for anti-(XOR) antibodies

Microtitre 96-well plates (Labsystems) were coated with either BXO or purified HXO in 0.1 M carbonate/hydrogen carbonate coating buffer, pH 9.6 (10 μg per ml, 100 μl per well) and incubated at room temperature for 2 hours or overnight at 4°C.

The plates were washed extensively with PBS-T and the wells were blocked with 1% (w/v) casein in PBS-T, 100 μl per well for 1 hour at room temperature or at 4°C overnight. The PBS-T/casein was removed and the plates were washed as before. The test antibody fraction - tissue culture supernatant or polyclonal antiserum - was added to the wells and doubling dilutions were made

across the plate, 100 μl per well. The plate was incubated for 2 hours at room temperature. After washing, the immobilized antibody was incubated with species-specific donkey anti-(mouse or rabbit immunoglobulin) antibody conjugated with HRP (Amersham International plc), diluted 1 in 1000 in PBS-T (100 μl per well) for 2 hours at room temperature. The plates were washed with PBS-T followed by PBS. Horseradish peroxidase substrate, 0.001% (w/v) tetramethyl benzidine [stock 10% (w/v) in dimethyl sulphoxide (DMSO)] and 0.006% (v/v) hydrogen peroxide in 0.05 M citrate/acetate buffer, pH 6.0, was added to the wells and the colour reaction was slowed with 1.84 M sulphuric acid (50 μl per well). The absorbance at 450 nm was measured using the Dynatech plate reader. Normal cloning medium was used as the negative control when screening hybridoma supernatant; for polyclonal antibody (pAb) screening, normal rabbit or mouse serum was used.

2.2.8 Coupling protein to cyanogen bromide-activated Sepharose

Biological molecules can be partially or wholly purified from a heterogeneous mixture by exploiting ligand/receptor affinities. By immobilizing either ligand or receptor on a gel or solid matrix, a re-usable affinity column can be constructed. This method of protein coupling was used to prepare HXO, BXO, protein A and monoclonal antibody (mAb) affinity columns (H2C2).

Cyanogen bromide-activated (CNBr) Sepharose 4B (Pharmacia Biotech Ltd) was suspended in 0.001 M HCl (200 ml), swilled gently and then left to swell for 15 minutes. The gel was washed on a sintered funnel with 0.001 M HCl (600 ml), distilled water (600 ml), and 0.1 M sodium hydrogen carbonate buffer,

pH 8.3 (600 ml). The protein to be coupled was dialysed against several changes of coupling buffer, 0.1 M sodium hydrogen carbonate buffer, pH 8.3, containing 0.5 M sodium chloride.

The protein was added to the gel at a ratio of 10 mg per ml swollen gel (3 g Sepharose 4B swelled to approximately 10 ml) when coupling protein A, and 5 mg protein per ml gel for all other proteins. The ligand and gel were tumbled end-over-end for 12 hours at 4°C. With HXO, the tumbling period was increased to 48 hours.

After coupling, the gel was centrifuged at 300 *g* for 10 minutes; the supernatant containing uncoupled ligand was retained for protein estimation. The gel was washed twice with coupling buffer, by dispersion and centrifugation The remaining active groups on the matrix were initially blocked with 0.2 M Tris, pH 8.0, for 2 hours, but this resulted in protein leaching from the Sepharose. The protocol was modified and 1 M ethanolamine, pH 8.0, was used to block the active sites. The gel was washed with 3 cycles of alternating pH (i.e. 0.1 M sodium acetate, pH 4.0, containing 0.5 M sodium chloride followed by 0.1 M Tris, pH 8.0, containing 0.5 M sodium chloride. The column was washed with PBS and stored in PBS containing 0.02% (w/v) sodium azide at 4°C.

2.2.8.1 Preparation of BXO affinity columns

Commercially available BXO (Biozyme Laboratories), 20 mg, was coupled with CNBr-activated Sepharose 4B at a ratio 5 mg per ml swollen gel (Materials and Methods 2.2.8).

Coupling efficiency was 85–93%, determined by estimation of uncoupled protein in the gel washes. The affinity matrix was packed in a column of suitable dimensions.

2.2.8.2 Preparation of HXO affinity columns

Construction of a column similar to that described for BXO, with 10 mg HXO, was attempted (Materials and Methods 2.2.8.1). The human enzyme was obtained from human breast milk (Appendix II). With 12 hours end-over-end mixing, coupling efficiency was low (i.e. less than 20% determined by estimation of uncoupled protein in the gel washes). On increasing the duration of mixing to 48 hours, the efficiency increased to 70%.

2.2.8.3 Preparation of protein A affinity columns

Protein A, 20 mg, was attached to CNBr-activated Sepharose at a ratio of 10 mg per ml swollen gel (Materials and Methods 2.2.8).

Coupling efficiency with this ligand was approximately 90% determined by estimation of uncoupled protein in the gel washes.

2.3 Antibody production, purification and labelling

2.3.1 Production of pAb

2.3.1.1 Immunization

Healthy rabbits were immunized with either 100 μg HXO purified from breast milk, or 100 μg BXO with an equal volume of alum adjuvant (Pierce).

Booster injections of the same immunogen were given 3–4 weeks later. Ten days after this, a small volume of blood was withdrawn from the rabbit and tested by a screening ELISA (Materials and Methods 2.2.7) to ensure that an immune response had been provoked.

If the immunization had been successful (i.e. titre $> 3 \times 10^4$), a further boost was given 3–4 weeks later. Approximately 20–30 ml blood was collected by bleeding the ear vein 10 days after the booster immunization. Subsequent boosts and bleeds were continued for 6 months.

2.3.1.2 Preparation of antiserum

The collected blood was allowed to stand at room temperature for 30 minutes. Using a scalpel or spatula, the clot was squeezed and chopped. The blood was left to stand at room temperature for a further 30 minutes, after which time it was centrifuged at 2500 *g* for 10 minutes. The serum was collected and stored at -20°C. Under these conditions, the polyclonal IgG was stable for at least 6 months.

2.3.2 Purification of pAb

2.3.2.1 Purification of IgG from antiserum

The clarity of the antiserum (V ml) was checked. If the sample was turbid, it was centrifuged at 2500 *g* for 15 minutes. Saturated ammonium sulphate solution (100%) was added to the antiserum, dropwise with constant stirring, to give 45% saturation. Stirring was continued for 15 minutes. After 30 minutes' centrifugation at 3000 *g*, the supernatant was discarded and the precipitate was redissolved in V/2 ml 0.2 M Tris-HCl, pH 8.0.

Saturated ammonium sulphate solution was added to the redissolved precipitate, dropwise with constant stirring, to give 40% saturation. Again, stirring was continued for 15 minutes and the precipitate was collected following centrifugation. The clean, white precipitate was redissolved in V/5 ml 0.03 M phosphate buffer, pH 7.3, and dialysed over 24 hours against two changes (5 l each) of the phosphate buffer.

The ammonium sulphate precipitation was followed by crude ion-exchange chromatography. A small column was packed with diethylaminoethyl-cellulose (DE-52; Whatman Ltd) equilibrated with 0.03 M phosphate buffer, pH 7.3. The column was washed thoroughly with the phosphate buffer, and the IgG preparation was loaded onto the column and eluted with phosphate buffer. Contaminants were eluted with the starting buffer and 0.5 M NaCl.

Although this method was used to obtain IgG in early experiments, it was later replaced by protein A-affinity chromatography (Materials and Methods 2.3.2.2).

2.3.2.2 Protein A-affinity purification of IgG from polyclonal antiserum

When a crude fraction of IgG was required, the high affinity of protein A (isolated from *Staphylococcus aureus)* for the Fc region of IgG was exploited. Protein A was immobilized on cyanogen bromide-inactivated Sepharose 4B as described previously (Materials and Methods; Section 2.2.8).

The pH of the antiserum was adjusted to 8.0 with 1 M Tris, pH 8.0. Antiserum was slowly recycled through a 4 ml protein A-Sepharose column, previously equilibrated with 0.01 M Tris, pH 8.0, overnight at 4°C.

The gel was washed with 0.1 M Tris, pH 8.0, and then with 0.01 M Tris, pH 8.0. The bound IgG was eluted with 0.1 M glycine, pH 3.0. Eluted fractions were collected in tubes containing 1/20 fraction volume 1 M Tris, pH 8.0. Fractions were pooled and dialysed against two changes of PBS.

2.3.2.3 BXO/HXO-affinity purification of polyclonal Ig from antiserum

Frozen antiserum was thawed at room temperature.

The chosen affinity column was equilibrated with either 0.01 M Tris buffer, pH 7.5, or PBS. The chromatography apparatus comprised peristaltic pump, UV monitor (UV-1), control unit (UV-1), chart recorder and fraction collector (Frac-100) in a dedicated system (Pharmacia Biotech Ltd). The A_{280} of the eluate was monitored and recorded.

Antiserum was diluted 1 in 10 in 0.1 M Tris, pH 7.5, or PBS, depending on the choice of equilibrating buffer, and recycled slowly through the column overnight at 4°C.

The column was washed extensively with the equilibrating buffer, and then with the same buffer containing 0.5 M sodium chloride. Bound antibodies were eluted with a cycle of low and high pH. Elution with 0.1 M glycine-HCl, pH 2.5, was followed by washing with 0.01 M Tris, pH 8.8, until the pH of the column reached 8.0. This was followed by elution with 0.05 M diethylamine, pH 11.5. The column was then washed with 10 mM Tris, pH 7.5, or PBS, and stored in 0.02% (w/v) sodium azide. Fractions containing antibody eluted at low pH were collected in tubes containing 1/20 fraction volume 1 M phosphate buffer, pH 8.0; fractions containing antibody eluted at high pH were collected in tubes containing 1/20 fraction volume 1 M phosphate buffer, pH 6.8.

The fractions were pooled according to pH of elution, dialysed against several changes of PBS and stored at either 4°C or with glycerol, 1:1 (v/v), at -20°C.

This protocol was subsequently simplified for the purification of anti-(BXO) antibodies on a BXO-affinity column. In this case, antibodies were eluted with 0.05 M diethylamine, pH 11.5, only; low pH did not appear to elute any antibodies. In contrast, when elution from an HXO affinity column was partially successful, bound proteins were eluted at both high and low pH.

2.3.3 Production of mAb

2.3.3.1 Normal cloning media

RPMI 1640 media (ICN Biomedicals Ltd)	
containing sodium hydrogen carbonate	(2 g/l)
Foetal calf serum (FCS), heat inactivated	
(Myoclone Plus; Gibco BRL)	10% (v/v)
Anti-PPLO agent (Gibco BRL)	1% (v/v)
L-glutamine (Gibco BRL)	0.002 M

Later batches included

Penicillin/streptomycin (Gibco BRL)	100 IU/ml

Batches of media were autoclaved at 20 lb/in^2 for 30 minutes and, after cooling to room temperature, were either used immediately or stored at -20°C for up to 6 months.

2.3.3.2 Immunization

Balb/c mice were immunized with 10–100 μg HXO from breast milk (Appendix II), with an equal volume of alum adjuvant.

Initially, an identical booster was given 3–4 weeks later, though later immunization schedules were altered to allow boosting 6–10 weeks after the initial immunization. A blood sample from the tail tip was taken 10 days after the boost and was tested by ELISA (Materials and Methods 2.2.7). If the anti-(XOR) titre was high compared with normal mouse serum, a final boost of 10 μg HXO with an equal volume of alum was given 3–4 weeks (or 6–10 weeks, in later schedules) after the second boost and the fusion was carried out 5 days later.

2.3.3.3 Myeloma cell line culture

Three weeks prior to the fusion, X-63 8Ag myeloma cells were continuously grown and sub-cultured. The cells were grown in cloning medium containing 0.13 mM 8-azaguanine before storage at -70°C and, on thawing, were grown in normal cloning medium and tested at intervals for aminopterin sensitivity. The cells were sub-cultured every 2–3 days in order to maintain log-phase growth.

2.3.3.4 Provision of a macrophage feeder cell layer

Macrophage feeder cell layers were prepared 1–2 days prior to the fusion. Balb/c mice were killed by cervical dislocation; fur and skin were sterilized by immersion in 70% ethanol. The skin was dissected back to expose the peritoneum. Sterile PBS (5 ml) was injected into the peritoneal cavity of each mouse. Mouse abdomens were massaged for several minutes to suspend the peritoneal macrophages in the PBS. These were then withdrawn by a pipette inserted through a hole cut in the peritoneum.

The macrophage suspensions were irradiated for 4 minutes (2000 rads) and pelleted by centrifugation, 10 minutes at 300 *g*. The supernatant was discarded and the cells were resuspended in a small volume of normal cloning medium. A cell count was performed and the macrophages were diluted to a suitable volume in cloning medium containing hypoxanthine, aminopterin and thymidine (HAT supplement) and seeded out in 96-well tissue culture plates (Falcon; Becton Dickinson Ltd) at approximately 20,000 per cm^2.

2.3.3.5 Fusion procedure and subsequent growth of hybridomas

Polyethylene glycol (PEG 4000; Serva), 4.5 g, was added to 5.5 ml PBS, pH 7.4, in a McCartney bottle. This was autoclaved and agitated while still hot to fully dissolve and disperse the PEG. The temperature of the PEG/PBS was maintained at 37°C until needed.

The immunized mouse was killed by cervical dislocation and the spleen was removed and transferred to a 90 mm Petri dish (Bibby Sterilin Ltd). The spleen cells were washed out with sterile PBS (10 ml) injected repeatedly into the spleen sac. The cells were transferred to a sterile tube and further dispersed by gentle trituration. The cell suspension was allowed to stand for a few minutes and any connective tissue settled out. The cells were transferred to a sterile glass centrifuge tube (50 ml).

The myeloma cells were harvested and approximately 2 x 10^7 cells were added to the glass centrifuge tube. A mouse spleen contains approximately 10^8 splenocytes and the ratio of myeloma cells to splenocytes in this fusion procedure was 1:5.

The mixed cells were centrifuged at 300 *g* for 10 minutes. The supernatant was discarded and the warm PEG/PBS solution (1 ml) was added, dropwise, over 60 seconds to the pellet, with constant agitation to prevent the cells clumping. This was immediately followed by adding warm PBS (1 ml) in the same manner. Further warm PBS was added slowly to reach a final volume of 20 ml. The cells were centrifuged as before, resuspended in warm normal cloning medium (20 ml) and transferred to a 50 ml sterile flask. The flask was incubated at 37°C overnight to allow completion of the fusion process.

The next morning the cells were centrifuged for 10 minutes at 300 *g*, and resuspended in cloning medium (100 ml) containing HAT supplement. The cells were dispensed into five macrophage-conditioned plates, 200 μl per well. The plates were incubated at 37°C (10% CO_2 in air) for 5 days.

The medium was replenished at day 5 by withdrawal of medium (150 μl) and replacement with fresh HAT medium (175 μl). After a further 5 days, the medium was similarly replenished with cloning medium supplemented with HT only. At day 15, the cells were again fed with HT medium.

Colonies appeared 2–3 weeks after the fusion and the supernatants were screened. Cells secreting antibodies positive on ELISA (Materials and Methods 2.2.7) were cloned.

2.3.3.6 Cloning by limiting dilution

The cells in a selected well were counted for viability and serially diluted in fresh normal cloning medium until the theoretical concentration was 100 cells per 15 ml. This volume of cells was dispensed over a freshly macrophage-conditioned 96-well plate (150 μl per well) giving an approximate theoretical ratio of 1 cell per well. The plates were incubated under normal conditions.

The screen/clone procedure was carried out three times after which the cell line was deemed to be monoclonal.

On several occasions, no hybridoma colonies formed following the first cloning stage. The initial cloning dilutions were subsequently altered to give theoretical ratios of 1 cell/well in 24 wells, 10 cells/well in 24 wells, 100 cells/well in 24 wells and 1000 cells/well in 24 wells. Cells from healthy colonies

at the lowest dilution were then grown up and cloned three times as previously described.

Monoclonal cell lines were established and the supernatant harvested at intervals; the supernatant was screened regularly to ensure antibody secretion had not stopped. Stocks were stored at -70°C.

2.3.3.7 Freezing hybridomas

Cells were carefully resuspended in the supernatant and centrifuged at 300 *g* for 10 minutes. Supernatant was removed and cells were resuspended in normal cloning medium (1 ml) and transferred to a 2 ml sterile cryogenic vial (Imperial Laboratories Ltd). The vial was placed on ice for 15 minutes. An equal volume of ice-cold freezing mixture [50% (v/v) FCS, 30% (v/v) RPMI 1640, 20% DMSO (v/v)] was added and the vial was mixed by inversion. The sealed vial was placed in the vapour phase of a liquid nitrogen storage vessel and was cooled at approximately 1°C per minute to -70°C. After 24 hours, the vial was fully immersed in the liquid nitrogen.

2.3.3.8 Thawing hybridomas

Cell lines were recovered from liquid nitrogen and rapidly defrosted by incubating the vials in a water bath at 37°C. The cells were washed with two changes of normal medium and returned to normal culture conditions.

2.3.4 Monoclonal antibody isotype ELISA

Rows of the microtitre plate were coated as indicated (Table 6). The 96-well microtitre plate was coated with 10 μg/ml BXO or HXO, or 1/1000 dilution isotype specific goat anti-(mouse Ig) antibody. The plate was incubated for 2 hours at room temperature, or 12 hours at 4°C.

96-well microtitre plate reference	Coating protein
A1-A12	HXO
B1-B12	BXO
C1-C12	α-IgG$_1$
D1-D12	α-IgG$_{2a}$
E1-E12	α-IgG$_{2b}$
F1-F12	α-IgG$_3$
G1-G12	α-IgA
H1-H12	α-IgM

Table 6. Antibodies used to coat the microtitre plate for monoclonal isotype ELISA.

The plate was washed thoroughly with PBS-T and free, non-specific binding sites were blocked by incubation with 1% (w/v) PBS-T/casein for 1–12 hours.

After further washing, test hybridoma supernatants were added, 100 μl/well, 1 supernatant/plate column. The plate was incubated for 2 hours at room temperature.

The wells were washed and incubated with donkey anti-(mouse Ig) antibody conjugated with HRP (Amersham International plc) for 2 hours.

The plate was washed and further rinsed with PBS; HRP substrate, 0.001% (w/v) tetramethyl benzidine [stock 10% (w/v) in DMSO] and 0.006% (v/v) hydrogen peroxide in 0.05 M citrate/acetate buffer, pH 6.0, was added to the wells and the colour reaction was slowed with 1.84 M sulphuric acid (50 μl per well). The absorbance at 450 nm was measured using the Dynatech plate reader.

2.3.5 Antibody labelling

2.3.5.1 Biotinylation of antibodies (Vector Laboratories' recommended protocol)

Biotin (long arm) N-hydroxysuccinimide ester (Vector Laboratories) was dissolved in DMSO, to give a final concentration between 25 and 50 mg/ml. The protein to be biotinylated, IgG or IgM at approximately 10 mg/ml, was dialysed against two changes of 0.1 M sodium hydrogen carbonate buffer, pH 8.5. Biotinylating reagent, 10% (w/w), was added dropwise to the protein solution. This was incubated, with gentle stirring, for 2 hours at room temperature. The

reaction was stopped by adding 10 mg glycine. Unreacted biotin was removed by extensive dialysis against PBS or by gel filtration through a 9.1 ml Sephadex G-25 M column (PD-10). Biotinylated antibody was stored at -20°C, 1:1 (v/v) with glycerol.

2.3.5.2 Determination of biotin/protein ratio (modified from Green, 1965)

A solution containing avidin 0.65% (w/v) in 0.02 M potassium phosphate buffer, pH 7.0 was prepared. The same buffer was used to prepare 0.005 M 4-hydroxyazobenzene benzoic acid (HBA). Avidin solution was carefully mixed with HBA in a cuvette at a ratio of 45:1. The absorbance was measured at 500 nm spectrophotometrically (Pye Unicam SP6-450 UV/VIS spectrophotometer; Philips). Samples (2 μl) of the biotinylated protein were added and the corresponding decrease in absorbance was determined. The assay was calibrated using sequential 1 μl additions of a standard biotin solution (0.20 mg/ml in 0.001 M sodium hydroxide).

This assay was also scaled down and carried out on a microtitre plate to conserve the biotinylated antibody.

A typical calibration curve is shown for this assay (Figure 8). Using this method, ratios of 6–7 biotin labels/IgG molecule and 2–3 biotin labels/IgM molecule were achieved.

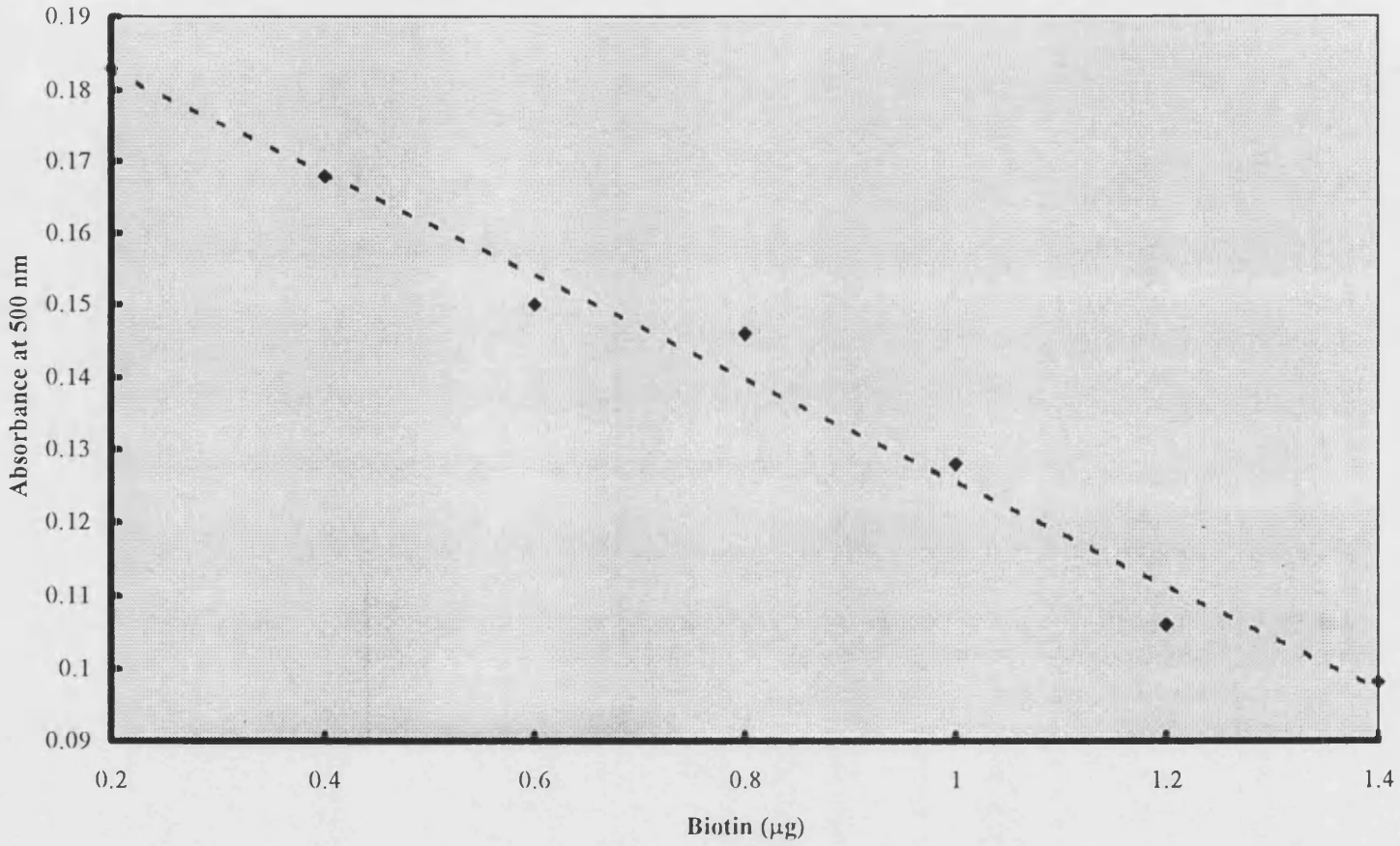


Figure 8. A sample reference curve for biotin/protein ratio determination using a method modified from Green (1965)

2.4 Cross-linking antibodies to protein A

Protein A was coupled to cyanogen bromide-activated Sepharose as described previously (Materials and Methods 2.2.8).

Rabbit affinity-purified anti-(BXO) pAb was mixed with the protein A gel (2 mg antibody per ml wet beads). The gel was incubated with the antibodies at room temperature for 1 hour with gentle rocking. The beads were washed twice with large volumes of 0.2 M sodium borate, pH 9.0, by centrifugation at 300 *g* for 10 minutes.

The beads were resuspended in the borate buffer, and enough dimethylpimelimidate was added to bring the final concentration to 20 mM. This suspension was gently rotated for 30 minutes at room temperature. The reaction was stopped by washing the beads in 0.2 M ethanolamine, pH 8.0, centrifuging and incubating for 2 hours in the same solution, at room temperature. The gel was washed in PBS and stored in PBS with 0.02% sodium azide at 4°C.

Elution of bound enzyme from this gel was with 0.05 M diethylamine, pH 11.5, containing 0.5 M sodium chloride. Fractions containing protein were collected in 1/20 volume 1 M phosphate buffer, pH 6.8. The gel was washed with 20 column volumes of 10 mM phosphate buffer, pH 8.0, between elutions.

2.5 Homogenization of animal and human tissue

N.B. Suitable precautions were taken when handling human postmortem tissue. All work was carried out in a Class II extraction hood, designated for use with human material.

Fresh or deep frozen tissue (thawed at room temperature) was chopped into small pieces. Tissue, 25% (w/v) in distilled water containing 4 mM dithiothreitol and protease inhibitors, leupeptin, antipain, aprotinin and pepstatin A (all at 1 μl/ml), was homogenized on ice. The crude homogenate was buffered with x 5 PBS, 10% (v/v) and centrifuged at 35,000 *g* for 30 minutes at 4°C. The pellet was discarded and finely ground ammonium sulphate was added to the supernatant (20 g/100 ml). The suspension was stirred at 4°C for 10 minutes and then centrifuged at 15,000 *g* for 30 minutes at the same temperature. The pellet was discarded and further ammonium sulphate was added to bring the final concentration to 40 g/100 ml. The suspension was stirred and centrifuged as before.

The pellet was suspended in PBS containing DTT and protease inhibitors as described for the homogenization solution, and dialysed against several changes of this PBS over 24 hours. The dialysed supernatant was filtered through a series of disposable filter units, 1.2 μm to 0.22 μm (Millipore UK Ltd) and used immediately.

2.6 Purification and activity of aldehyde oxidase

2.6.1 Purification of aldehyde oxidase from rabbit liver (Warne and Stell, 1990)

Fresh rabbit liver was homogenized, 25% (w/v) in ice-cold 0.067 M phosphate buffer, pH 7.8. The homogenate was incubated at 55°C for 10 minutes and rapidly cooled in an ice bath.

The cooled homogenate was centrifuged at 15,000 *g* for 45 minutes at 4°C. The pellet was discarded and finely ground ammonium sulphate was added, 40 g/100 ml. The suspension was stirred slowly in an ice bath for 20 minutes; the precipitated protein fraction was collected by centrifugation at 6000 *g* for 20 minutes at 4°C.

The pellet was resuspended in homogenization buffer (1 ml/10 g original weight) and then equilibrated with Buffer A (0.1125 M glycine-NaOH, pH 9.0 with 0.1125 M sodium chloride, 10^{-4} M EDTA and 0.002 M cysteine), either by dialysis or gel filtration (Sephadex G-25M; PD-10).

A 5 ml *p*-aminobenzamidine-Sepharose 6B column was equilibrated with Buffer A. Crude homogenate was recycled through the column for 12 hours at 4°C. The column was washed with Buffer A until the A_{436} of the eluate returned to baseline (benzamidine absorbs at 280 nm). Bound protein was eluted with Buffer B (Buffer A with 0.006 M benzamidine).

Eluted fractions with positive A_{436} were pooled and precipitated with 65% (w/v) ammonium sulphate followed by centrifugation at 6000 *g* for 20 minutes. The pellet was resuspended in 100 μl Buffer A, injected onto a Superose 6 gel

filtration column (FPLC: Pharmacia Biotech Ltd), and eluted with 0.05 M bis-Tris propane-HCl, pH 7.2 containing 1 x 10^{-4} M EDTA and 2 x 10^{-3} M cysteine.

The fraction containing protein was determined from the UV A_{280} trace and injected onto a Mono Q anion exchange column. Elution with a linear sodium chloride gradient (0–35% in 0.02 M bis-Tris propane-HCl, pH 6.9 containing 5% (v/v) betaine and 2 x 10^{-3} M cysteine) was unsuccessful and the protein could not be retrieved from the column.

Fractions from different stages in the purification method were analysed by SDS-PAGE (Materials and Methods 2.2.3) and assayed for XOR and aldehyde oxidase activity (Materials and Methods 2.2.5, 2.6.2).

From visual interpretation of the SDS-PAGE pattern, it appeared that gel filtration and anion exchange chromatography were unnecessary for the purity required and were omitted from later purifications.

The purification was repeated and the fractions were tested by anti-(BXO) capture ELISA (Results 3.2.2.3) and Western Blot with IgM anti-(XOR) mAb (Materials and Methods 2.2.4).

2.6.2 Spectrophotometric assay for aldehyde oxidase activity (Stell *et al.*, 1989)

The rate of *p*-dimethylaminocinnamaldehyde oxidation by aldehyde oxidase was measured spectrophotometrically. The assay mixture comprised *p*-dimethylaminocinnamaldehyde 2.5 x 10^{-5} M in 0.067 M potassium phosphate buffer, pH 7.0; the reaction was monitored at 398 nm. The extinction coefficient for the substrate at 398 nm is 3.05 x 10^{4} $M^{-1}cm^{-1}$.

3 Results

3.1 Antibody production, purification and labelling

NB Titre values correspond to the dilution of antibody preparation at which absorbance is half maximum, and are expressed numerically as the reciprocal of that dilution.

3.1.1 Polyclonal antibody preparation

Two rabbits, coded 117 and 124, were immunized with HXO prepared from human breast milk (Appendix II); rabbit coded 118 was immunized with BXO. Stocks of antisera from previously immunized rabbits (Dr A Rogers), were also available.

A sample of blood was withdrawn from the rabbit 10 days post-immunization, from which antiserum was prepared (Materials and Methods 2.3.1.2). This was tested by ELISA against HXO and/or BXO, immobilized on microtitre plates (Materials and Methods 2.2.7). Figure 9 shows typical titration curves from two ELISAs, one with 10 μg/ml BXO immobilized on the microtitre plate and the other with 10 μg/ml HXO immobilized on the microtitre plate. As a negative control, normal rabbit serum (NRS) was also tested on each plate. The titres from these ELISAs are summarized in Table 7.

Both HXO and BXO were found to be highly immunogenic in rabbits, with antiserum titres against BXO and HXO consistently greater than 2.75×10^4.

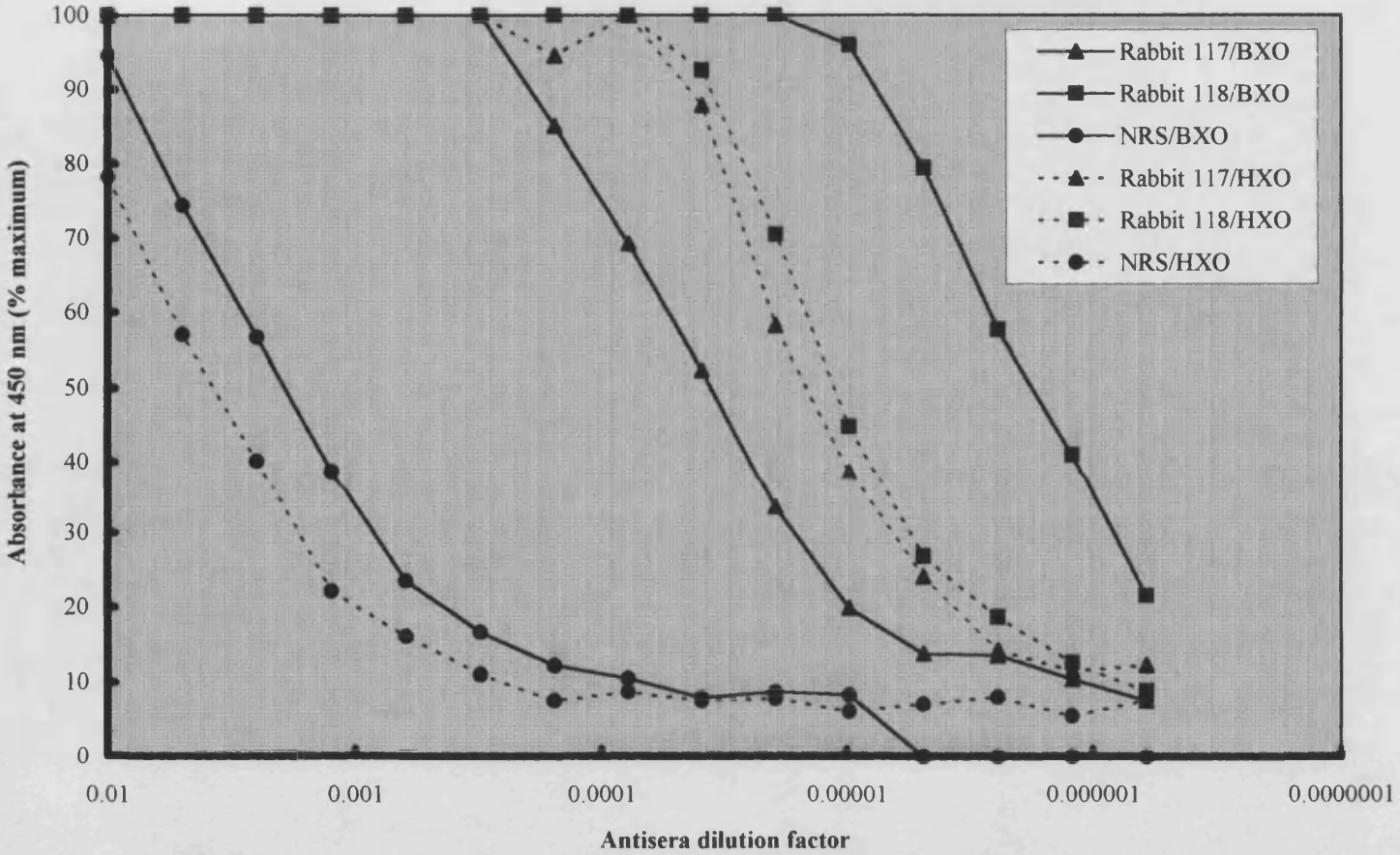


Figure 9. Titration curves of two samples of antiserum obtained 10 days post-immunization with HXO (117) or BXO (118). One microtitre plate had 10 μg/ml BXO immobilized and the second plate was coated with 10 μg/ml HXO.

Immobilized enzyme						
	HXO			BXO		
Rabbit (immunogen)	118 (BXO)	117 (HXO)	NRS	118 (BXO)	117 (HXO)	NRS
Titre x 10^4	9.09	6.67	0.026	58.8	2.78	0.048

Table 7. Antisera titration curves from rabbits designated 117 and 118 compared; one microtitre plate was coated with 10 μg/ml BXO and the other with 10 μg/ml HXO.

3.1.1.1 Purification of IgG from antiserum

Ammonium sulphate precipitation followed by crude ion-exchange chromatography (Materials and Methods 2.3.2.1) yielded 6–8 mg IgG per ml antiserum. Being relatively time-consuming, however, this method was soon replaced by protein A purification (Materials and Methods 2.3.2.2).

3.1.1.2 Protein A purification of IgG from antiserum

Buffered antiserum was recycled through a protein A column and bound IgG was eluted with 0.1 M glycine pH 3.0 (Materials and Methods 2.3.2.2). Approximately 0.1 g IgG was routinely prepared from 10 ml antiserum.

3.1.1.3 Affinity purification of anti-BXO antibodies

Antiserum, diluted 1 in 10 in 0.1 M Tris, pH 7.5, or in PBS, depending on the choice of buffer used to equilibrate the affinity column, was recycled slowly through the BXO affinity column overnight at 4°C. Elution with cycles of high and low pH buffers (Materials and Methods 2.3.2.3) yielded negligible protein at low pH and was, therefore, replaced by single-step elution with 0.05 M diethylamine only. Affinity purification using BXO gel routinely yielded 10–18 mg polyclonal antibody from 20 ml anti-(BXO) antiserum, on elution with 0.05 M diethylamine (Table 8; Figure 10).

Affinity-purified anti-(BXO) pAb was used to probe BXO and HXO on Western blots (Materials and Methods 2.2.4). Photograph 1 shows an SDS-PAGE gel and subsequent detection pattern achieved with affinity-purified anti-(BXO) pAb (from rabbit 118 in this case) and denatured HXO and BXO. As expected, the BXO lanes show strong staining, with major bands staining at M_w 136, 126, 99, 64 and 43 kDa and many minor bands also staining. There was also cross-reaction with HXO, with major enzyme bands at 141 and 136 kDa showing strongest staining, and further detection at 225, 194, 104, 71 and 61 kDa.

In addition, BXO affinity-purified anti-(BXO) pAb had higher titres against BXO than HXO in ELISA (Materials and Methods 2.2.7). Examples of curves and titres are shown (Figure 11; Table 9).

Elution cycle	Total protein in eluted fractions (mg)					
	Rabbit 78	Rabbit 78	Rabbit 79	Rabbit 79	Rabbit 118	Rabbit 118
1	5.1	5.5	11.7	9.3	4.3	6.8
2	3.0	3.1	4.4	6.1	4.8	5.4
3	1.8	2.0	0.9	2.3	3.2	2.9
4	-	0.8	-	0.5	1.2	0.6
Total	9.9	11.4	17.0	18.2	13.5	15.8

Table 8. Antiserum (20 ml) from rabbits immunized with BXO (rabbit identification number indicated) was recycled through a 10 ml BXO affinity column. Bound protein was eluted with 0.05 M diethylamine, pH 11.5. The unadsorbed fraction was recycled and the elution was repeated until it was estimated that negligible anti-(BXO) antibody remained in the unadsorbed fraction.

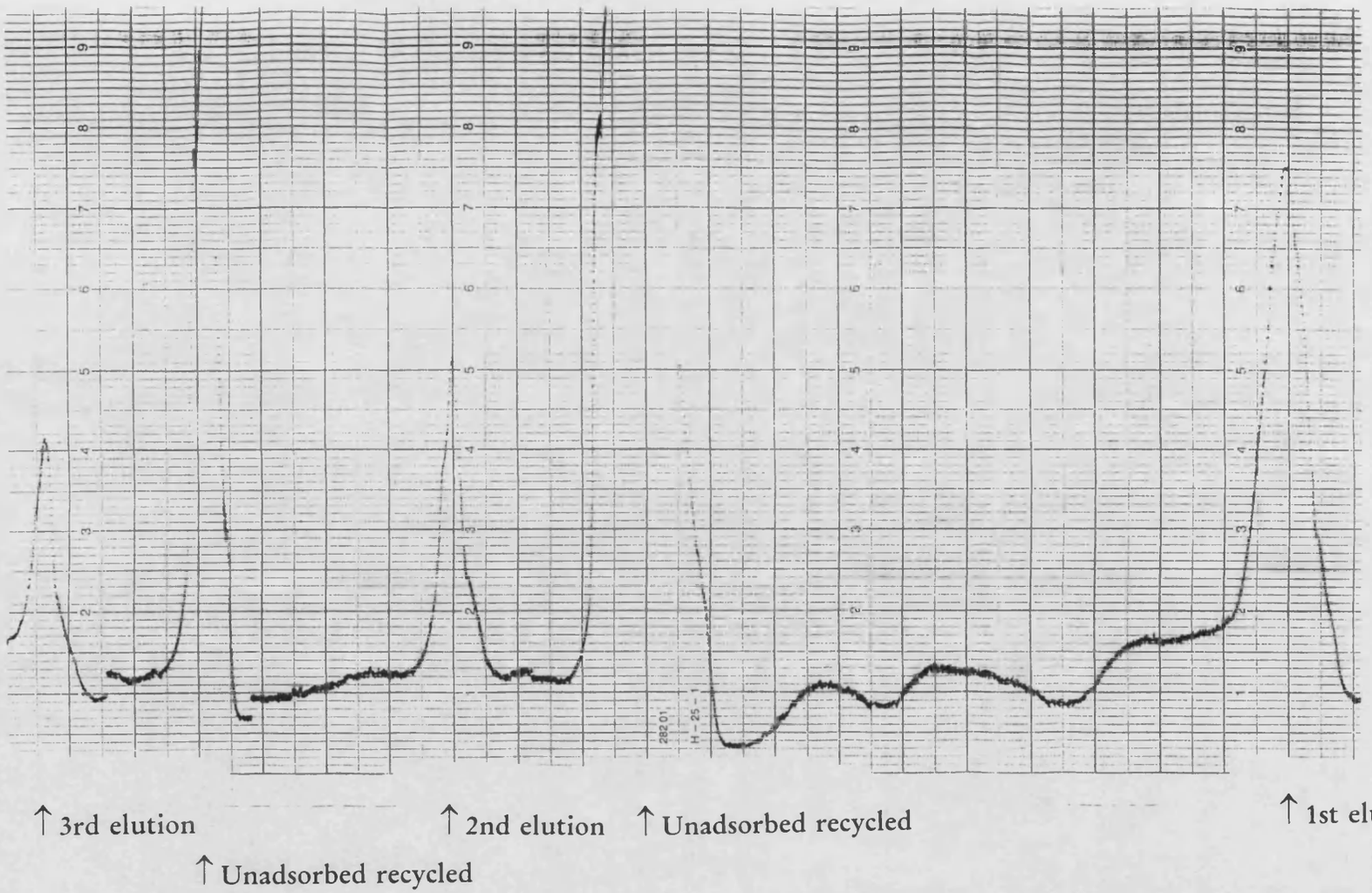
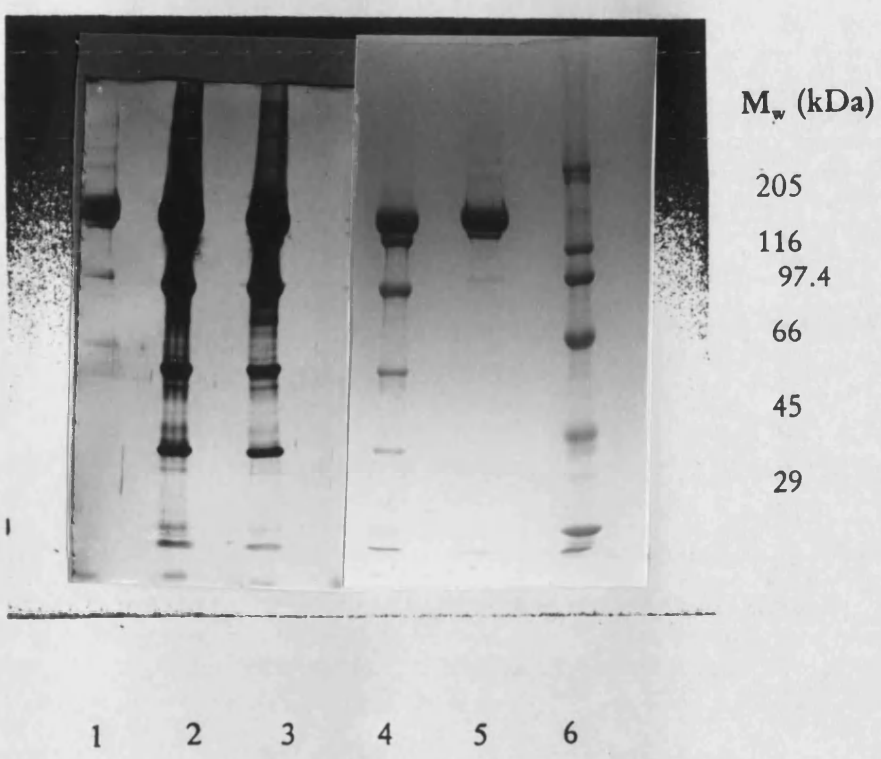


Figure 10. Elution profile of anti-(BXO) pAb from the BXO affinity column.



Photograph 1. Western blot with affinity-purified rabbit anti-(BXO) polyclonal antibodies (118 at 50 μg/ml) and corresponding SDS-PAGE gel.

1. HXO (Western blot)
2. BXO (Western blot)
3. BXO (Western blot)
4. BXO (SDS-PAGE)
5. HXO (SDS-PAGE)
6. Molecular weight markers

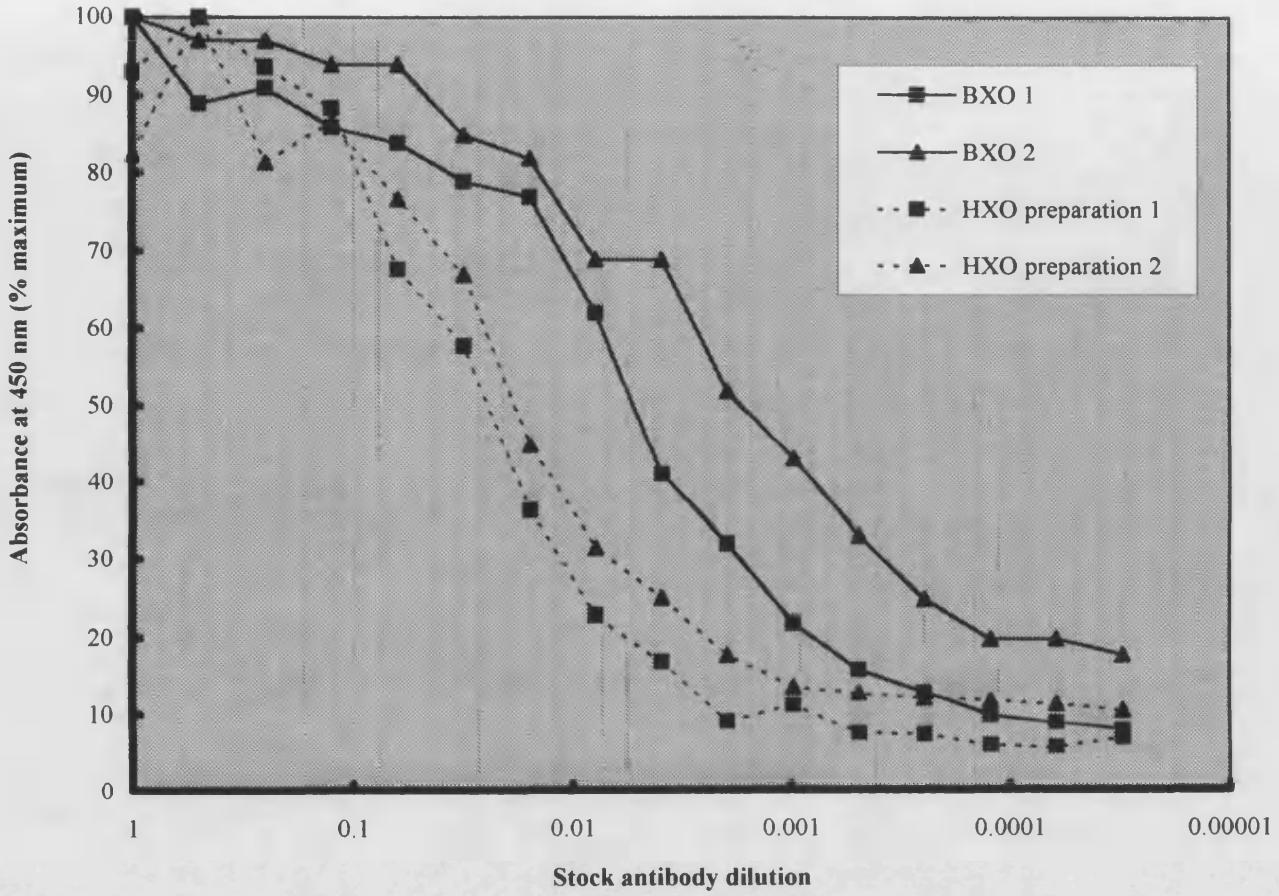


Figure 11. Titration curves of affinity-purified anti-(BXO) pAb from rabbit 118 against two preparations of HXO from breast milk (HXO preparation 1, HXO preparation 2), commercially available BXO (BXO 1) and BXO prepared from fresh bovine milk in the laboratory (BXO 2). Titres are summarized in Table 9.

	Immobilized enzyme			
	HXO preparation 1	HXO preparation 2	BXO 1 (Biozyme)	BXO 2 (in-house preparation)
Titre	1.92×10^2	5.88×10^2	3.12×10^3	8.3×10^3

Table 9. Titres of affinity-purified anti-(BXO) pAb from rabbit 118 against two preparations of HXO from breast milk (HXO preparation 1, HXO preparation 2), commercially available BXO (BXO 1) and BXO prepared from fresh bovine milk in the laboratory (BXO 2).

3.1.1.4 Affinity purification of anti-HXO antibodies

Affinity purification of anti-(HXO) pAb using an HXO affinity column was less efficient than anti-(BXO) pAb purification using a BXO affinity column (Results 3.1.1.3). Enzyme breakdown appeared to cause rapid deterioration of the affinity column as evidenced by the following experiment.

Beads were removed from the HXO affinity column after one attempted purification (i.e. 5 days after column preparation). These were subjected to SDS-PAGE (Materials and Methods 2.2.3) using a 'minigel' system (Bio-Rad Laboratories Ltd). Although, using this system, the band resolution is poor, the enzyme appeared to have broken down to fragments running with M_w approximately 96, 82, 63 and 31 kDa (Photograph 2). Protein bands corresponding to HXO monomers (M_w ~ 150 kDa) are absent from the gel. A comparison lane shows BXO from a BXO affinity column that had been prepared a month previously and used to affinity purify several batches of

antiserum. In this case, the band pattern characteristic of BXO is retained, though some lesser degree of breakdown does appear to have occurred. A typical HXO breakdown pattern on storage at 4°C for 5 days is shown (Photograph 3)

The instability and paucity of purified HXO made routine preparation and use of HXO affinity columns impractical. Results from the two purifications performed are presented, however (Table 10).

Photograph 4 shows a Western blot with a batch of affinity-purified anti-(HXO) pAb (rabbit 117) and HXO. The enzyme (lane 3) had been purified (i.e. post-gel filtration stage, refer to Appendix II) approximately 10 days prior to use and showed a pattern of enzyme breakdown on SDS-PAGE. Although the antibodies detected bands at around 140–125, 80 and 75 kDa, strongest staining occurred at bands corresponding to approximately 53–57 and 25–27 kDa.

The antibodies were also used to detect proteins in a sample of crude HXO [i.e. post-ammonium sulphate precipitation stage, refer to Appendix II (lane 1)]; the staining pattern is similar to that of the purified HXO, with bands at 140, 136, 80, 56 and 27 kDa.

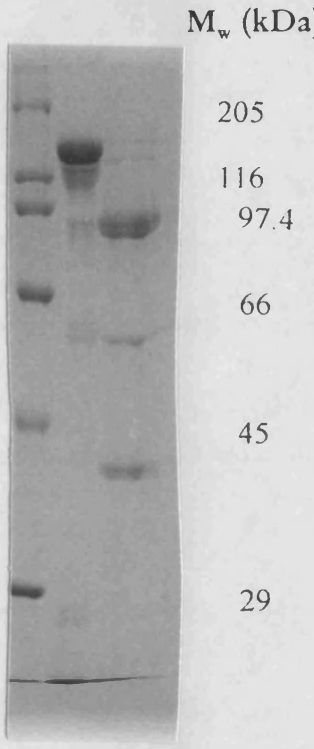
A sample of a non-HXO protein often copurifying with the enzyme and running with a molecular weight of 80 kDa was included in this blot [(lane 2) see also Results 3.3.4.2]. Although detection of protein running with this molecular weight occurred with both the crude and purified HXO, staining of this band was very weak.

HXO affinity-purified anti-(HXO) pAb had similar titres against BXO and HXO. Figure 12 shows titration curves of affinity-purified anti-(HXO) pAb from rabbit 117 against two preparations of HXO from breast milk (HXO preparation 1, HXO preparation 2), commercially available BXO (BXO 1) and BXO prepared from fresh bovine milk in the laboratory (BXO 2). Titres are summarized in Table 11.

1 2

Photograph 2. Comparison of HXO removed from HXO-affinity column at day 5 after preparation, with BXO removed from a BXO-affinity column at day 30 after preparation.

1. BXO removed at day 30
2. HXO removed at day 5

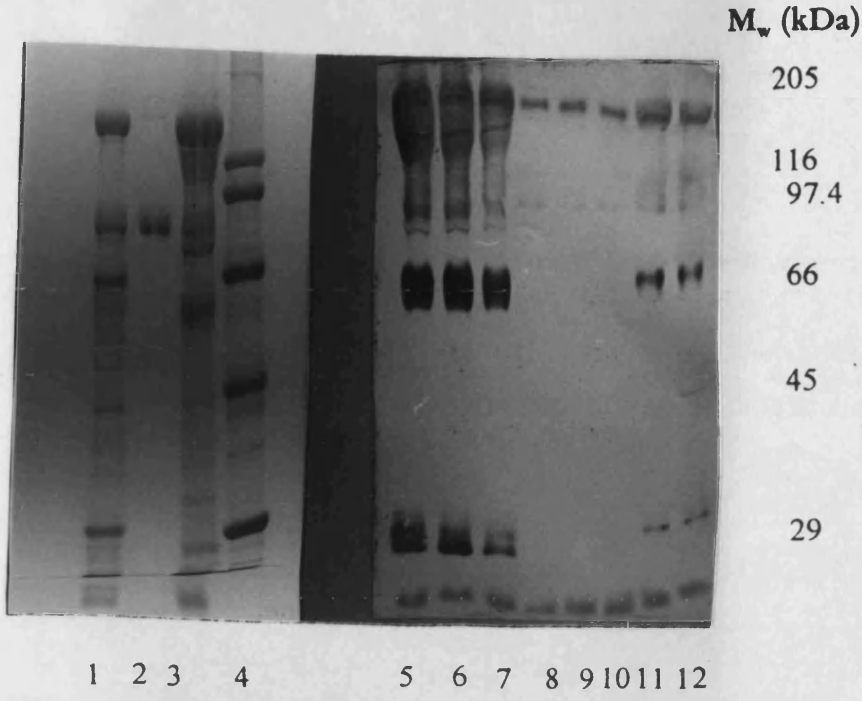


Photograph 3. Comparison of freshly purified HXO with HXO at day 5 after preparation.

1. Molecular weight markers
2. Freshly purified HXO
3. HXO at day 5 after preparation

Eluent	Cycle	Total protein in eluted fractions (mg)	
		Rabbit 117	Rabbit 124
0.1 M glycine, pH 2.5	1	2.1	1.6
0.05 M diethylamine, pH 11.5	1	1.5	0.9
0.1 M glycine, pH 2.5	2	2.7	1.2
0.05 M diethylamine, pH 11.5	2	0.3	0.5
0.1 M glycine, pH 2.5	3	0.5	negligible
0.05 M diethylamine, pH 11.5	3	negligible	negligible
Total		7.1	4.2

Table 10. Antiserum (10 ml volumes) from rabbits immunized with HXO (rabbit identification number indicated) were recycled through 10 ml HXO affinity columns. Bound protein was eluted with 0.1 M glycine, pH 2.5, and 0.05 M diethylamine, pH 11.5. The unadsorbed fraction was recycled and the elutions were repeated until it was estimated that negligible anti-(HXO) antibody remained in the unadsorbed fraction.



Photograph 4. SDS-PAGE gel and corresponding Western blot with affinity-purified rabbit anti-(HXO) polyclonal antibodies (117 at 50 μg/ml).

1. Partially purified HXO (SDS-PAGE)
2. 80 kDa contaminant (SDS-PAGE)
3. Purified HXO at 10 days after preparation (SDS-PAGE)
4. Molecular weight markers (SDS-PAGE)

5–7. Purified HXO at 10 days after preparation (Western blot)

8–9. 80 kDa contaminant (Western blot)

11, 12. Partially purified HXO (Western blot)

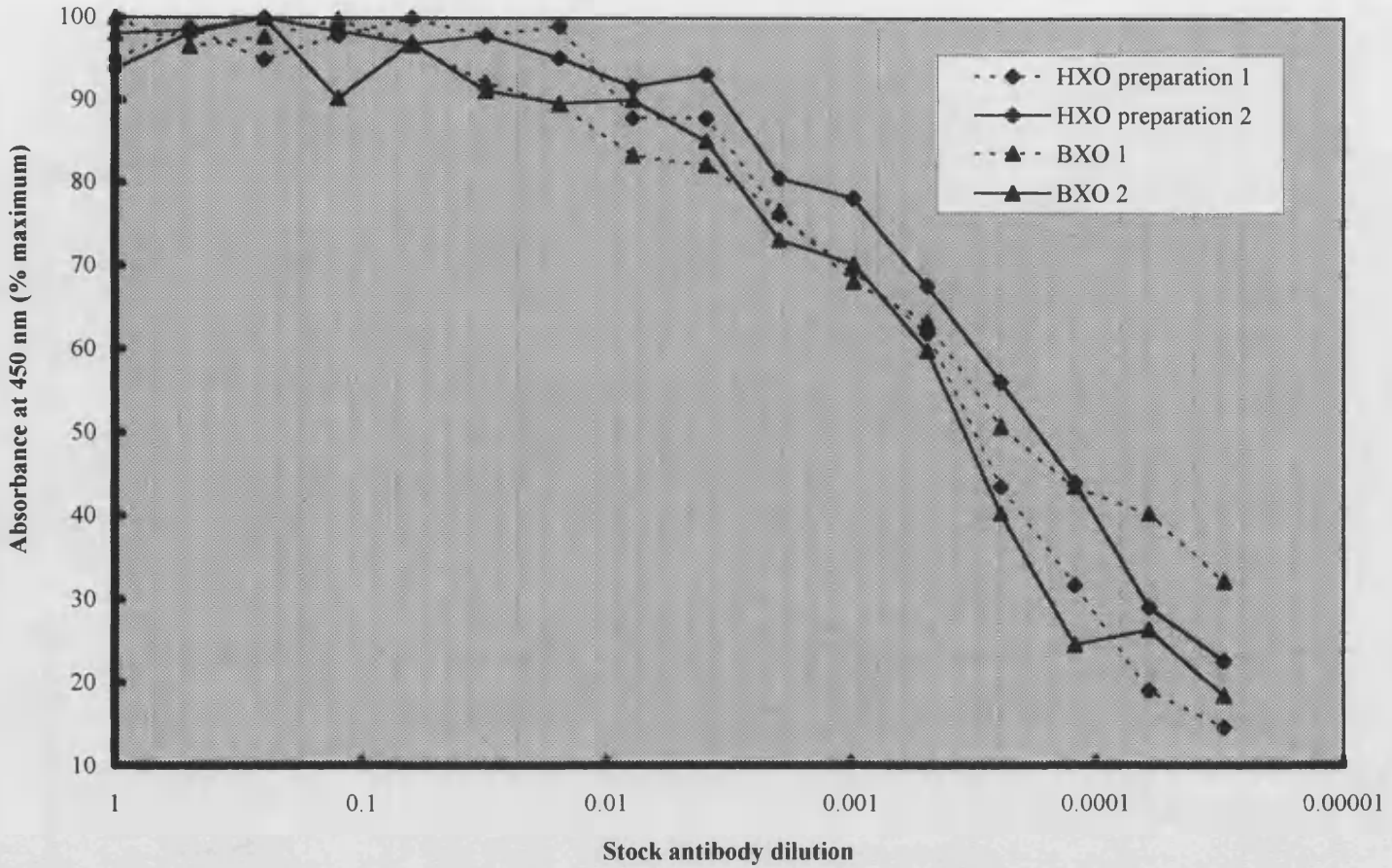


Figure 12. Titration curves of affinity-purified anti-(HXO) pAb from rabbit 117 against two preparations of HXO from breast milk (HXO preparation 1, HXO preparation 2), commercially available BXO (BXO 1) and BXO prepared from fresh bovine milk in the laboratory (BXO 2). Titres are summarized in Table 11.

	Immobilized enzyme			
	HXO preparation 1	HXO preparation 2	BXO 1 (Biozyme)	BXO 2 (in-house preparation)
Titre	4.54×10^3	2.94×10^3	3.22×10^3	5.88×10^3

Table 11. Titres of affinity-purified anti-(HXO) pAb from rabbit 117 against two preparations of HXO from breast milk (HXO preparation 1, HXO preparation 2), commercially available BXO (BXO 1) and BXO prepared from fresh bovine milk in the laboratory (BXO 2).

3.1.2 Monoclonal antibody production

A mouse was immunized with HXO (Materials and Methods 2.3.3.2), and antiserum, obtained 10 days post-immunization, was diluted and titrated across a microtitre plate coated with BXO or HXO (Materials and Methods 2.2.7). An example of a titration curve is shown (Figure 13; titre = 5.55×10^3).

After several immunization boosts, judged successful by ELISA titre (i.e $> 5 \times 10^3$), the immunized mouse was sacrificed by cervical dislocation. The fusion procedure for hybridoma production was followed (Materials and Methods 2.3.3.5); healthy hybridoma colonies were screened for anti-(XO) Ig production, by the standard ELISA (Materials and Methods 2.2.7), and class tested (Materials and Methods 2.3.4).

Hybridoma supernatants with anti-(XOR) activity, judged by ELISA end product ($A_{450} > 0.2$ above reference blank) indicated cell lines for expansion and cloning. The monoclonal cell lines established are summarized in Table 12.

Photographs 5 and 6 show a typical SDS-PAGE band pattern for HXO and BXO, and Western blot detection pattern using an anti-(XOR) mAb (2G3 in this case). All the monoclonal antibodies raised cross-reacted with HXO and BXO on Western blots.

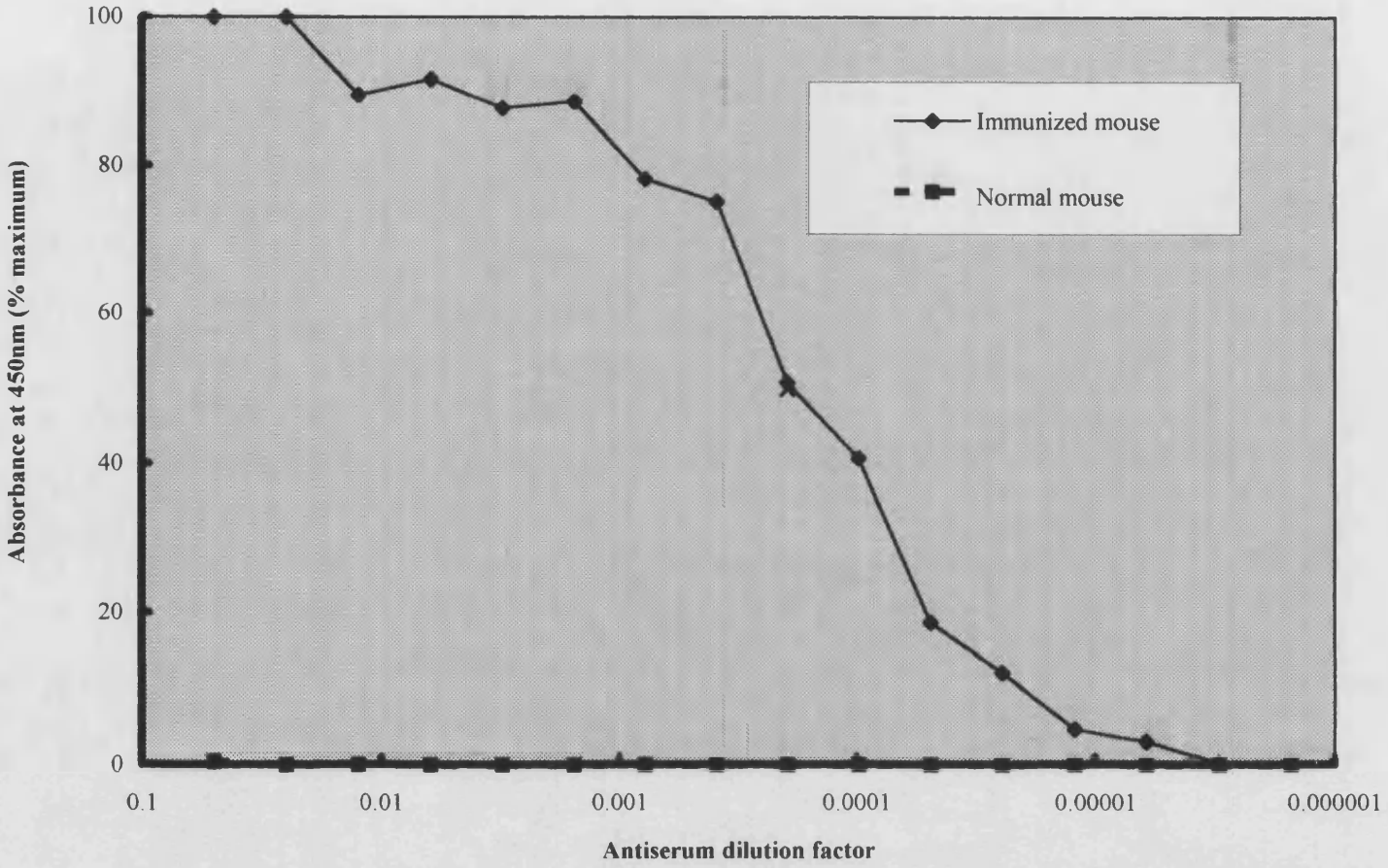
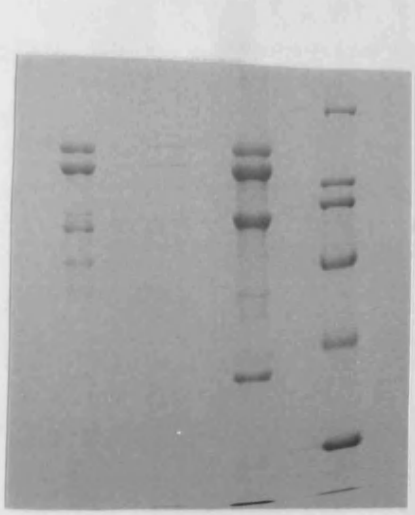


Figure 13. ELISA comparing the anti-BXO titration curves of antiserum from a mouse immunized with BXO with normal mouse serum.

Hybridoma	Immunogen	Ig class	anti-HXO	anti-BXO
*H3G7	HXO	IgM	+	+
*B5B4	BXO	IgM	+	+
*H2C2	HXO	IgG	-	-
2A3	HXO	IgM	+	+
2G3	HXO	IgM	+	+

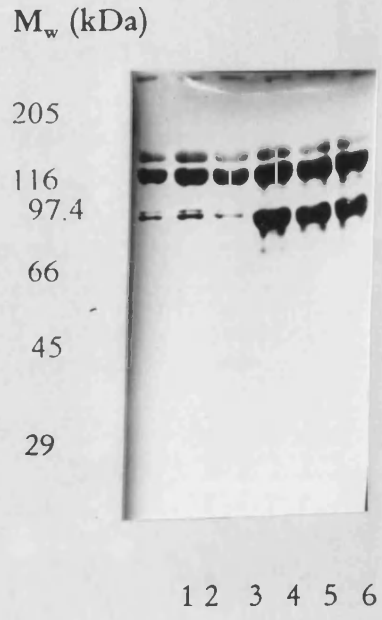
Table 12. Five hybridoma cell lines secreting monoclonal antibodies were established (*fusion performed by Dr A Rogers).



1 2 3 4

Photograph 5. SDS-PAGE band patterns for HXO and BXO.

1. Partially purified HXO
2. Purified HXO
3. BXO
4. Molecular weight markers



Photograph 6. Corresponding Western blot, using supernatant from cell line 2G3.

1-3. Partially purified HXO

4-6. BXO

Four fusions were attempted prior to those resulting in the establishment of hybridoma lines 2A3 and 2G3. On the first two occasions, the primary fused cells became infected and were discarded. As a result, antibiotics (penicillin/streptomycin 100 IU/ml; Materials and Methods 2.3.3.6) were added to all batches of cloning medium.

On the subsequent two occasions, hybridomas resulted from the fusion but did not survive the first cloning. The initial cloning procedure was subsequently modified (Materials and Methods 2.3.3.6).

3.1.3 Determination of the H2C2 antigen

Supernatant from the hybridoma line H2C2 did not show positive staining on Western blot analysis, though it did have a titre comparable with that of the other anti-(XOR)-positive supernatants on HXO ELISA (i.e. ~50). In order to gain information about H2C2 recognition, an affinity chromatography column was constructed with the monoclonal antibody as the ligand.

Solid ammonium sulphate was added to H2C2 supernatant to give 45% saturation. The solution was stirred for 30 minutes at room temperature and centrifuged at 3000 *g* for 30 minutes. The supernatant was discarded and the pellet was resuspended in 0.1 M sodium hydrogen carbonate buffer, pH 8.3, containing 0.5 M sodium chloride to give a final concentration of ~5 mg/ml. The solution was dialysed against two changes of 0.1 M sodium hydrogen carbonate buffer, pH 8.3, containing 0.5 M sodium chloride.

The dialysed mAb solution was coupled with CNBr-activated Sepharose (Materials and Methods 2.2.8), packed into a small column and equilibrated with

PBS. Crude HXO (post-ammonium sulphate precipitation; Appendix II) was exchanged into PBS via gel filtration using a 9.1 ml Sephadex G-25 M column (PD-10; Pharmacia Biotechnology Ltd) and a sample (1 ml) was loaded onto the H2C2 affinity column. After washing with PBS, bound protein was eluted with 0.05 M diethylamine containing 0.5 M NaCl, pH 11.5. The diethylamine-eluted fraction was exchanged into PBS via gel filtration, assayed for HXO activity (Materials and Methods 2.2.5) and subjected to SDS-PAGE (Materials and Methods 2.2.3).

The gel and subsequent Western blot with HXO-affinity-purified anti-(HXO) pAb are shown in an earlier section (Photograph 4, Results 3.1.1.4). The diethylamine-eluted fraction comprised an 80 kDa protein, with a trace of protein running with molecular weight approximately 140 kDa. On Western blot with HXO-affinity-purified pAb, the 80 kDa band stained very weakly. The HXO activity of the eluted fraction was negligible as determined by spectrophotometric assay.

H2C2 supernatant was tested by ELISA against the enzyme stock used to immunize the parent mouse (stored at -20°C: HXO 1) and a newer enzyme stock prepared using a modified purification protocol (HXO 2; Appendix II). The results are shown (Figure 14). The H2C2 titre against HXO 1 is comparable with that of the other (IgM) mAb-secreting hybridoma supernatants (45). However, coloration with HXO 2 was weak and a titration curve was not achieved.

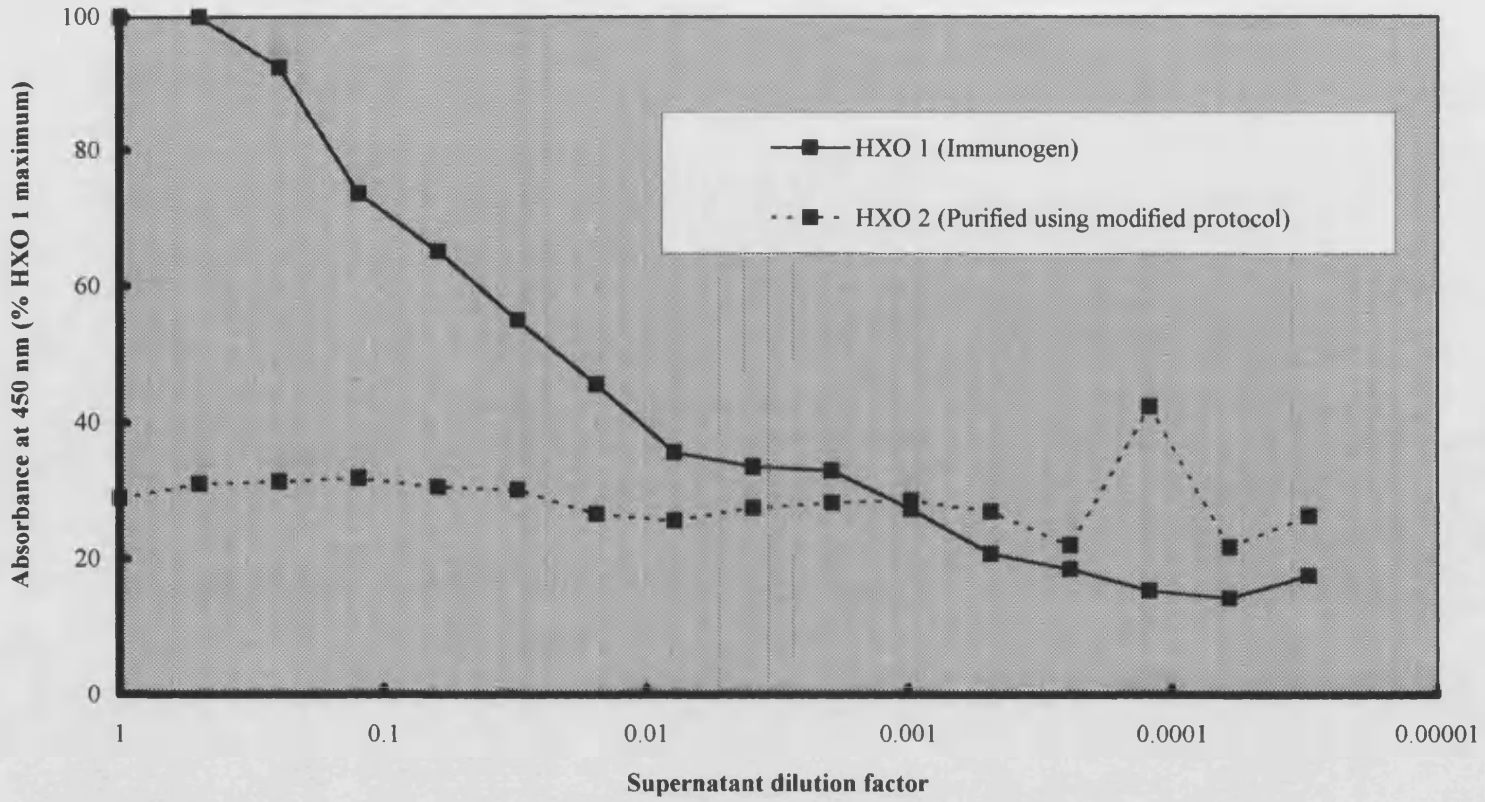


Figure 14. Titration curves of H2C2 supernatant against the enzyme stock used to immunize the parent mouse (HXO 1) and a newer enzyme stock prepared using a modified purification protocol (HXO 2; Appendix II).

3.2 Development of ELISA to measure HXO and BXO

One of the main aims of this project was to develop a method of quantifying XOR protein in biological mixtures. Different ELISAs were attempted, using either the anti-(XOR) IgM mAb or the anti-(XOR) pAb. It was hoped that, on establishment of a workable assay, monoclonal IgG(s) could be substituted for the monoclonal IgM or polyclonal IgG.

3.2.1 Inhibition ELISA

By their nature, the anti-(XOR) IgM monoclonal antibodies were more specific than the anti-(XOR) polyclonal antibodies (compare Photographs 1 and 4 with Photograph 6). These antibodies were, therefore, first choice for use in an ELISA.

As a result of their pentameric nature, IgM antibodies do not, in general, coat microtitre plates effectively. An ELISA was designed using BXO as the plate coat; mAb was incubated with the test sample, which contained BXO, and then titrated across the microtitre plate. The difference between absorption with and without incubation with test sample related to antigen in the test sample. Again, the pentameric nature of IgM required that the amount of antibody in the incubation mixture be kept minimal so that saturation of paratopes was possible. Suitable dilutions of H3G7 were determined empirically to be between 1/500 and 1/1000.

Microtitre 96-well plates were coated with BXO in 0.1 M carbonate/hydrogen carbonate coating buffer, pH 9.6, (5 μg per ml, 100 μl per well) and incubated at room temperature for 2 hours or overnight at 4°C .

The plates were washed thoroughly with PBS-T and non-specific protein-binding sites were blocked by incubation with PBS-T/casein (2 hours).

During this time, a range of volumes of anti-(HXO) mAb H3G7 was mixed with a constant volume of BXO and the antigen/antibody mixtures were allowed to stand for 1 hour.

The microtitre plates were washed and 100 μl aliquots from each mixed and incubated sample were titrated (doubling dilutions) across the plate. As a positive control, aliquots from mixtures with PBS in place of BXO were titrated across the plate; as a negative control, samples of BXO at the same concentration (v/v) as in the BXO/mAb mixtures were also titrated across the plates.

The plates were incubated at room temperature for 2 hours, and then washed thoroughly with PBS-T. Wells were incubated with species-specific goat anti-(mouse Ig) Ig/HRP conjugate (Amersham International plc) at stock dilution 1/1000, 100 μl per well for 2 hours at room temperature.

Plates were washed thoroughly with PBS-T and rinsed with PBS. Horseradish peroxidase substrate - 0.05 M citrate/acetate buffer, pH 6.0, with 0.001% (w/v) tetramethyl benzidine [stock 10% (w/v) in DMSO] and 0.006% (v/v) hydrogen peroxide - was added to the wells and the colour reaction was slowed with 1.84 M sulphuric acid (50 μl per well). The absorbance at 450 nm was measured as described previously.

By altering the length of antigen-antibody incubation and the amount of BXO immobilized on the microtitre plate, it was possible to detect a range of BXO concentrations (Figure 15). However, because the IgM antibodies were of low affinity (Appendix III), only very shallow reference curves were achieved. As useful reference curves in ELISA should not extend beyond a 2 to 3 log scale (Kemeny, 1991) and in view of the poor reproducibility of this assay, an alternative was sought.

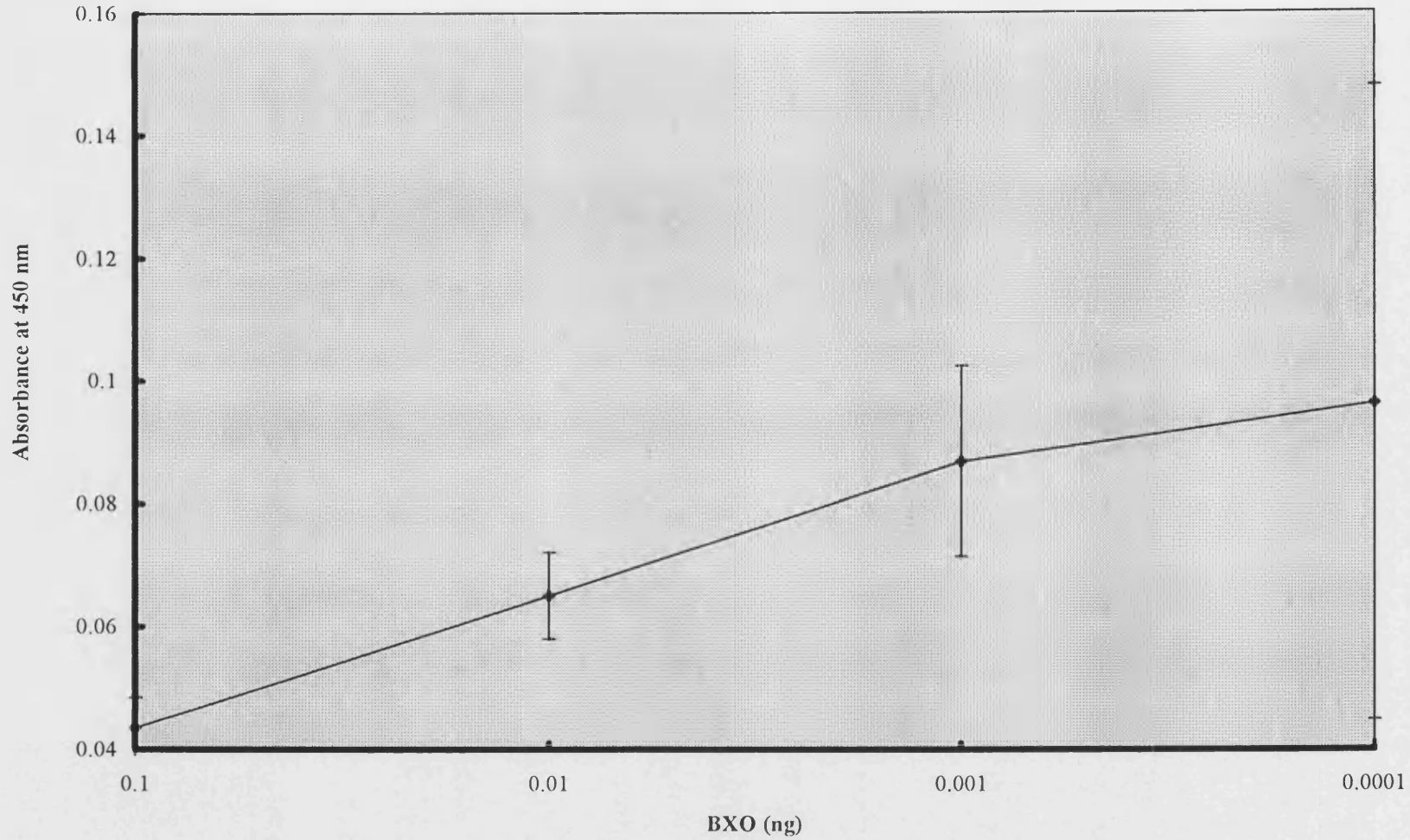


Figure 15. Sample calibration curve with BXO, using the anti-(BXO) inhibition ELISA, n=8.

3.2.2 Capture ELISAs for the detection of BXO and HXO

3.2.2.1 Combining monoclonal and polyclonal antibodies

A successful capture ELISA requires that the primary and detector antibodies recognise different antigen epitopes. A heterogeneous pAb population is likely to recognise a variety of epitopes, in contrast to a mAb population which is homogeneous in nature. As the mAbs were murine in origin, and the pAbs were obtained from immunized rabbits, it was possible to combine these in a capture ELISA, using a species-specific, enzyme-labelled tertiary antibody as detector.

Affinity-purified anti-(BXO) polyclonal antibody was immobilized on the microtitre plate (5 μg/ml). Three stock solutions of BXO in PBS (50 μg/ml, 5 μg/ml and 0.5 μg/ml) were titrated across the plate (doubling dilutions) in duplicate. As a negative control, PBS replaced the BXO in a further row (negative control 1); the final row had dilution from 50 μg/ml BXO for subsequent incubation with PBS in place of secondary antibody (negative control 2).

The wells were all incubated with B5B4 (1/1000 dilution in PBS), except the control row (negative control 2), which was incubated with PBS. After washing, the wells were incubated with species-specific goat anti-(mouse) Ig conjugated with HRP, adsorbed against rabbit Ig (Amersham plc). Subsequent steps are as described in Materials and Methods 2.2.7.

All wells developed high background coloration, except those in the control row (negative control 2), and there was no significant difference between results obtained using no antigen (negative control 1) and those with BXO at 50 μg/ml. This indicated that an association between the primary and secondary

antibody was occurring. The coloration could not be due to recognition between tertiary and primary antibody as it did not occur in the absence of the secondary antibody (negative control 2).

Similar high background values occurred when the mAb was used to coat the plate, and the pAb was used as secondary antibody [with species-specific goat anti-(rabbit) antibody conjugated with HRP as tertiary antibody]. However, in this case, titration curves with BXO diluted from 50 μg/ml and 5 μg/ml were achieved (Figure 16).

From these results it appeared that either the pAb was cross-reacting with the mAb, or native antigen was associated with the affinity-purified pAb, causing high background in the absence of BXO in the assay mix.

As the sensitivity of the assay could not be improved below around 30 ng BXO (Figure 16), alternative combinations were tested incorporating the biotin/avidin system.

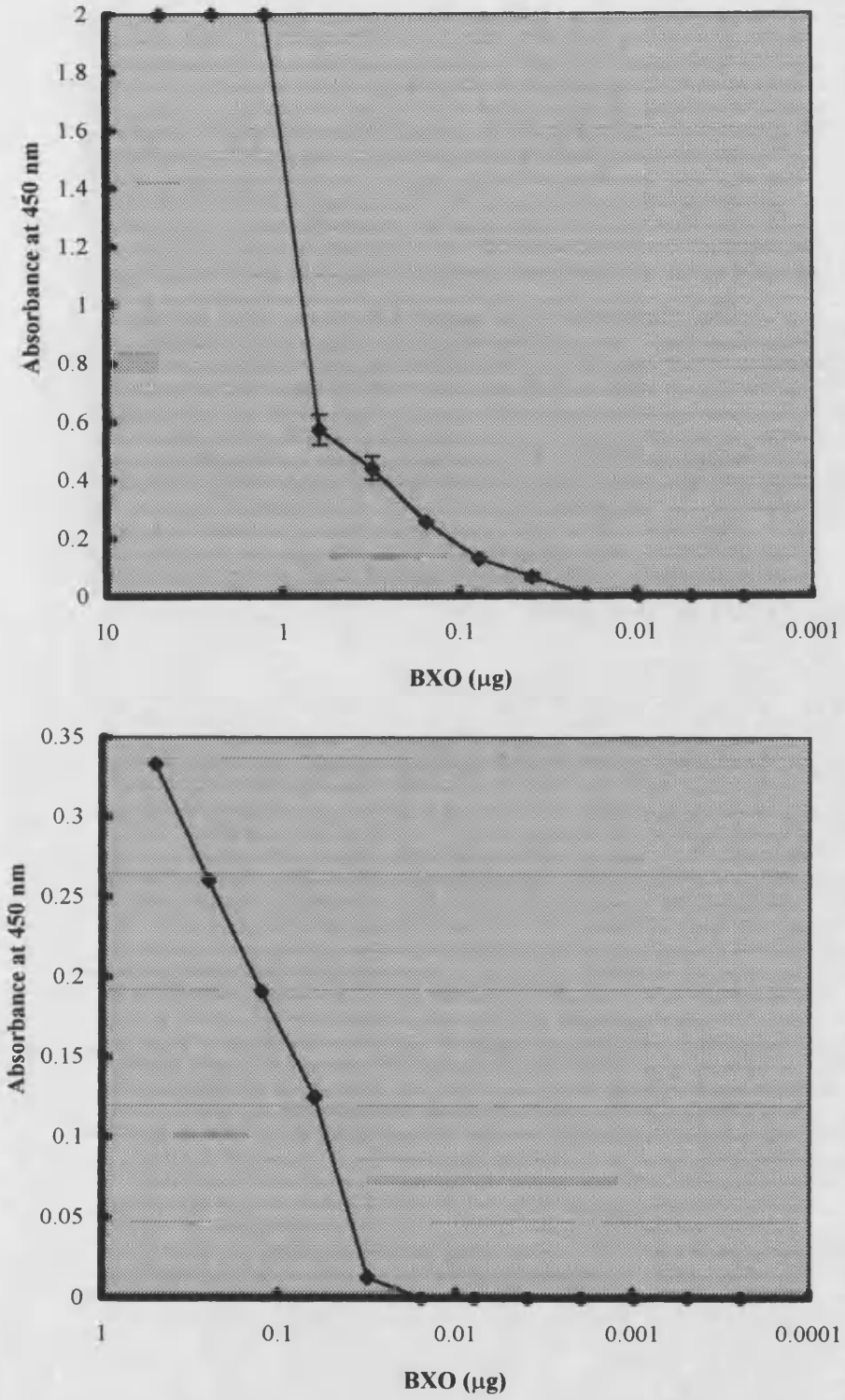


Figure 16. Sample curves with BXO, using a mAb/pAb capture ELISA. Monoclonal antibody B5B4 was used to coat the plate, and anti-(BXO) pAb was used as secondary antibody [with species-specific anti-(rabbit) antibody conjugated with HRP as tertiary antibody].

3.2.2.2 Using IgM mAbs

Although the similarities in behaviour between the different IgM mAbs indicated that they recognised the same epitopes, it seemed a worthwhile exercise to try to incorporate two different mAb populations into a capture ELISA.

Both H3G7 and B5B4 were biotinylated (Materials and Methods 2.3.5.1). Non-biotinylated H3G7 was used to coat the plate, while biotinylated B5B4 was used as the detector, and *vice versa*. Streptavidin (or later, as a cheaper alternative, Extravidin) conjugated with HRP was used in place of the tertiary antibody conjugate described previously (Results 3.5.2.1).

No coloration above background was achieved using either combination. This was most probably the result of recognition of the same epitopes by the different IgM antibodies, though inefficient coating of the microtitre plate and low affinity of both IgM lines could be contributing factors.

As the 'sticky' Fc portions of the IgM molecule are not exposed, IgM antibodies coat microtitre plates less well than IgG antibodies. In order to expose the Fc portions and, hence, make the coating antibody more 'sticky', fragmentation (reduction followed by gel filtration; Johnstone and Thorpe, 1990) was attempted. However, coating the plate with IgM fragments did not improve the assay, and coloured end-point was not achieved.

Similarly, coating the microtitre plate with goat anti-(mouse IgM) antibody prior to incubation with the IgM fraction, did not affect the results.

3.2.2.3 Using polyclonal antibodies only

As neither of the IgM mAbs investigated appeared suitable for incorporation in to an ELISA, investigation of the less desirable pAb/pAb capture ELISA was made. By incorporating a label in one fraction of the antibody, this fraction can be used as the detection antibody with the same antibody, non-labelled, used as the capture antibody. Subsequent detection is via the label.

The following assay used a biotin label attached to IgG prepared from anti-(XOR) antiserum, in conjunction with affinity-purified anti-(XOR) antibody from the same batch of antiserum. Detection is via streptavidin or Extravidin conjugated to HRP.

Once a high-affinity, IgG mAb has been established, it could then be substituted for the biotinylated (secondary) polyclonal IgG fraction.

Polyclonal IgG was prepared from anti-(BXO) antiserum using protein A chromatography (Materials and Methods 2.3.2.2). Approximately half of the IgG prepared was then passed through a BXO affinity column, yielding affinity-purified anti-(BXO) IgG (Results 3.1.3). The non-specific polyclonal IgG fraction was biotinylated (Materials and Methods 2.3.5.1) to give 6–7 biotin labels/IgG molecule).

Microtitre plates were coated with affinity-purified anti-(BXO) pAb (10 μg/ml; 100 μl per well) for 12 hours at 4°C or 2 hours at room temperature. The wells were washed with PBS-T and incubated with 1% (w/v) PBS-T/casein for 1 hour at room temperature. The test sample, PBS with BXO, was incubated in the wells for 2 hours at room temperature. The wells were washed and incubated with biotinylated pAb, diluted to an empirically determined optimum

dilution in PBS-T (100 μl per well), for 2 hours at room temperature. The wells were washed and incubated with streptavidin or Extravidin, conjugated to HRP, diluted 1 in 1000 in PBS-T, (100 μl per well), for 1 hour at 37°C. The wells were washed with PBS-T and PBS. Horseradish peroxidase substrate - 0.05 M citrate/acetate buffer, pH 6.0 with 0.001% (w/v) tetramethyl benzidine [stock 10% (w/v) in DMSO] and 0.006% (v/v) hydrogen peroxide - was added to the wells and the colour reaction was slowed with 1.84 M sulphuric acid (50 μl per well). The absorbance at 450 nm was measured as described previously (Materials and Methods 2.2.7).

The graph from this ELISA was approximately linear between 6 and 14 ng BXO (Figure 17). The sensitivity of the assay was slightly extended by substituting Extravidin-alkaline phosphatase for Extravidin-HRP (Figure 18). After incubation with Extravidin-alkaline phosphatase, *p*-nitrophenyl phosphate (200 μl/well) was added for approximately 30 minutes. The colour reaction was slowed with 3 M sodium hydroxide, and the absorbance of each well at 405 nm was measured spectrophotometrically. However, as the enzyme label did not make a significant difference, and HRP reagents were commonly used in-house, Extravidin-HRP was routinely used in this ELISA.

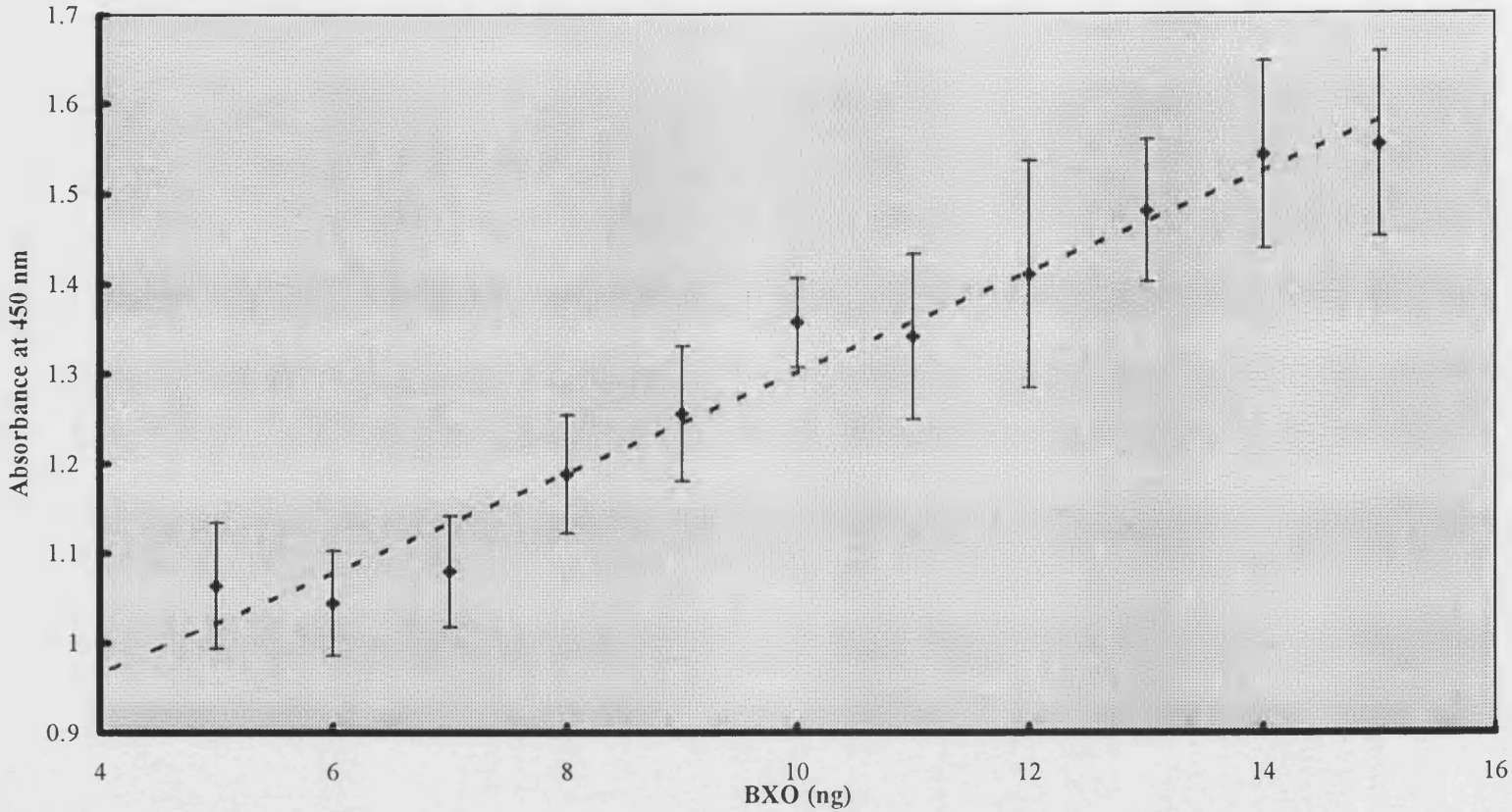


Figure 17. Calibration curve for BXO using a pAb/pAb capture ELISA with the biotin/avidin-HRP detection system, n=8.

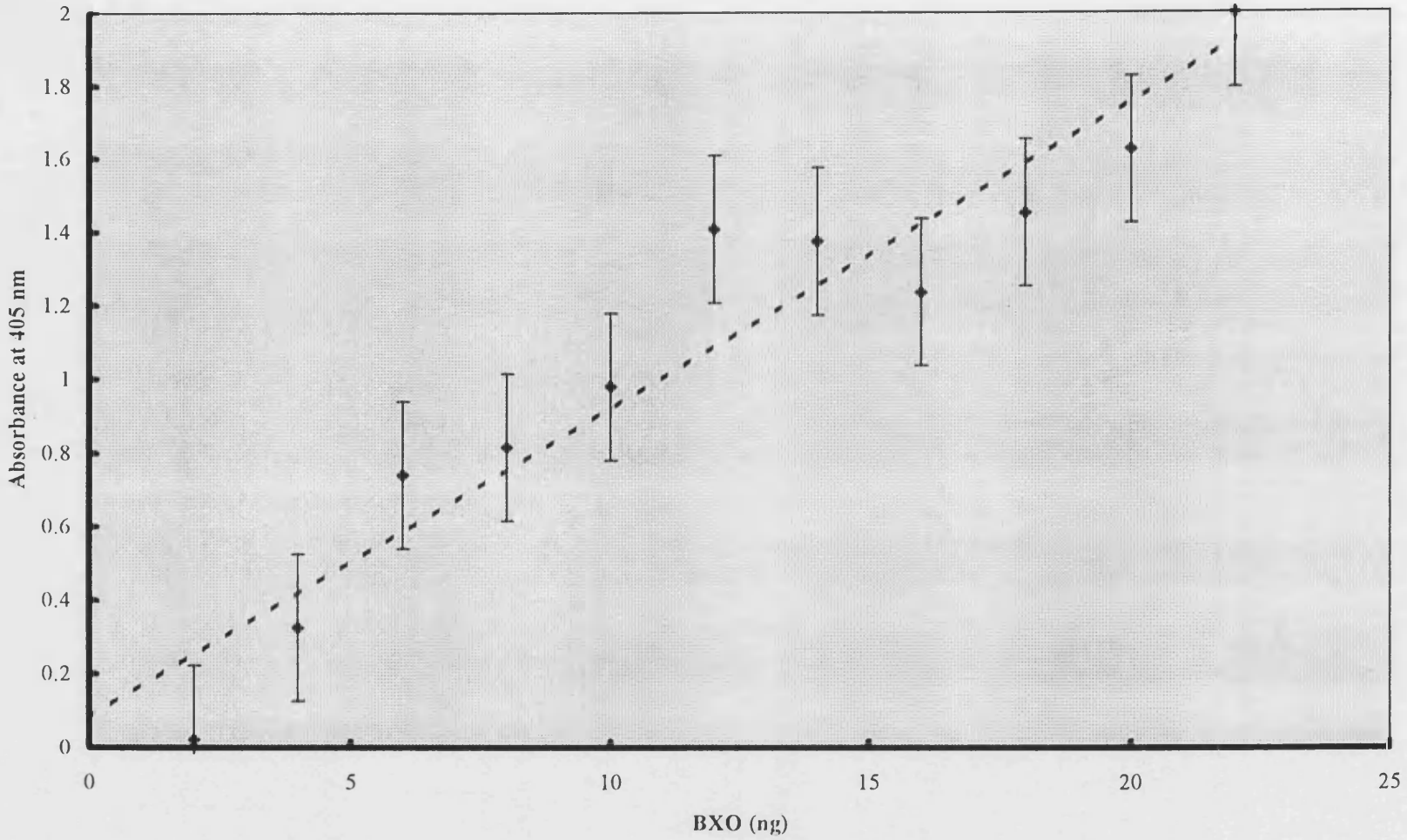


Figure 18. Calibration curve for BXO using a pAb/pAb capture ELISA with the biotin/avidin-alkaline phosphatase detection system, n=8.

By substituting anti-(HXO) IgG - affinity purified and non-specific - for anti-(BXO), detection of HXO in PBS was achieved (Figure 19). It is interesting to note that substituting HXO for BXO in the ELISA using anti-(BXO) IgG was unsuccessful, with no end-point coloration being achieved. This was probably due to the lower affinity of these antibodies for the human enzyme.

Initial standard curves achieved in this ELISA could not, however, be reproduced using later batches of antibodies. High, uniform end-stage coloration resulted, even in the absence of XOR. As the two antibody phases were prepared from the same batch of antibody, anti-(idiotope) reaction was unlikely. It appeared, therefore, that antigen was present, in excess amounts, in the assay. Simple gel filtration of a batch of affinity-purified antibody indicated that the polyclonal antibodies existed in two states (Figure 20). From the results of the later ELISAs, the gel filtration trace probably represents free and complexed antibodies.

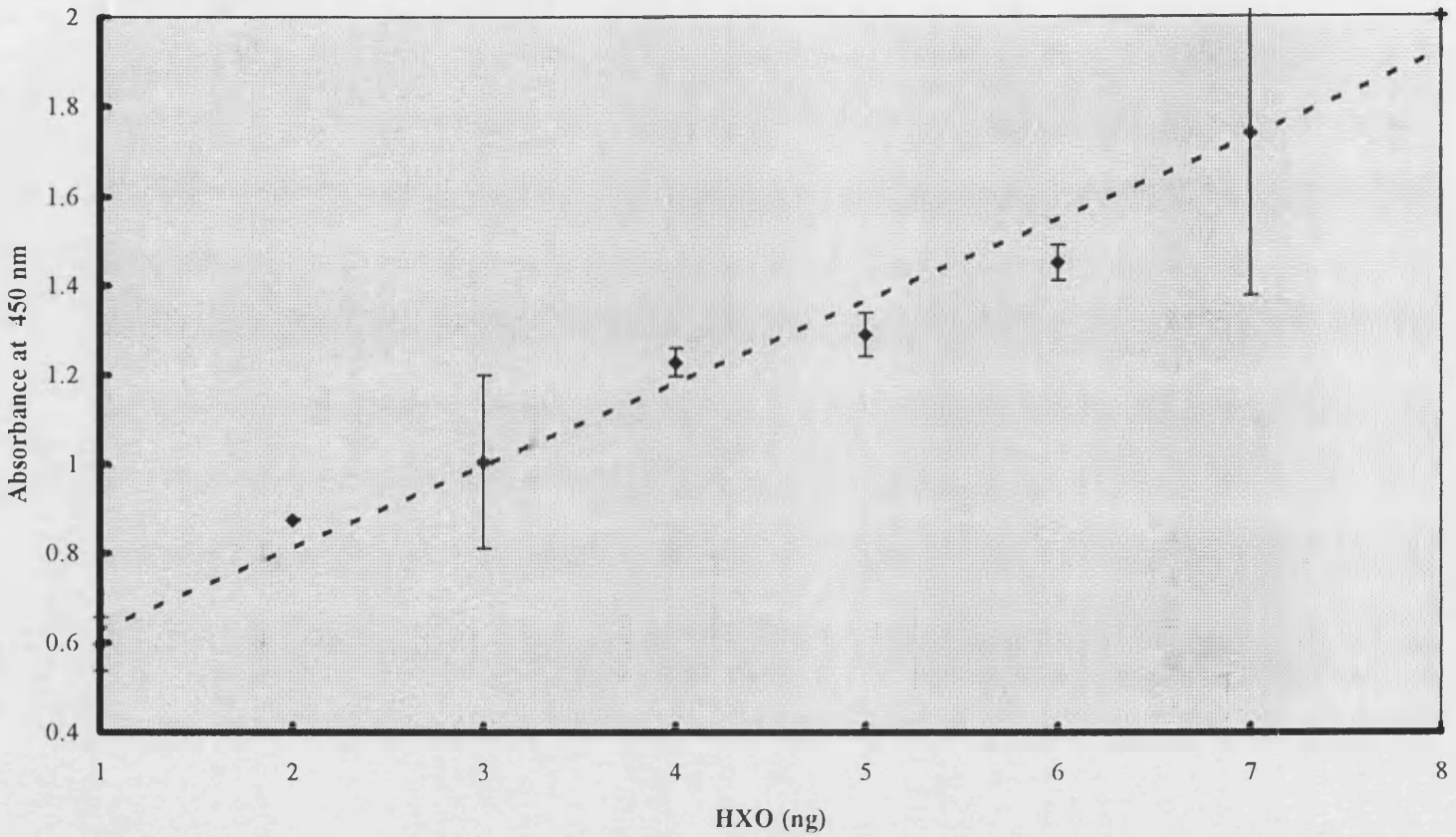


Figure 19. Calibration curve for HXO using a pAb/pAb capture ELISA with the biotin/avidin-HRP detection system, n=8.

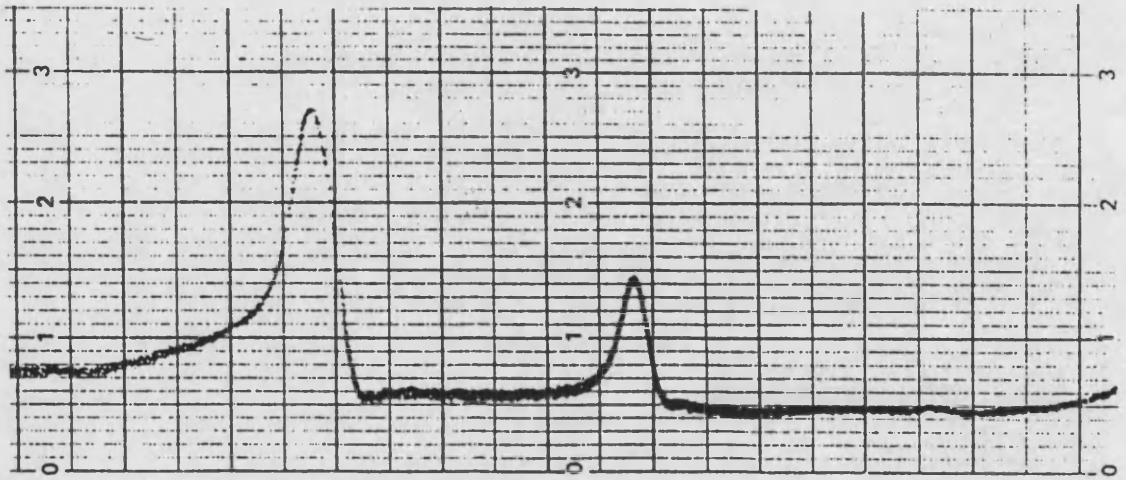


Figure 20. Gel filtration trace (UV detection at 280 nm) showing, on the basis of size, two distinct populations of pAb existed in a standard preparation from antiserum.

3.3 Partial purification of BXO and HXO

As mouse anti-(BXO/HXO) monoclonal IgG would be of potential use in purifying the enzyme from heterogeneous mixtures, preliminary experiments were designed using rabbit affinity-purified anti-(BXO) pAb.

Rabbit anti-(BXO) pAb, affinity purified against BXO, were cross-linked to protein A/Sepharose 4B using dimethylpimelimidate (Materials and Methods 2.4). Efficiency of cross-linking was assessed by determining the protein content of the gel washes. Several gels were prepared and the efficiency of coupling was 70–85%.

3.3.1 Extraction of BXO from BXO in solution

The matrix was packed into a 3 ml column and pre-elution with PBS and 0.5 M sodium chloride was performed to remove weakly bound protein. A solution of BXO in PBS (5 ml at 0.5 mg/ml) was slowly recycled through the column for 12 hours at 4°C. After washing with 20 column volumes of 10 mM phosphate buffer, pH 8.0, bound enzyme was eluted with 0.05 M diethylamine, pH 11.5, containing 0.5 M sodium chloride. Fractions containing protein were collected in 1/20 volume 1 M phosphate buffer, pH 6.8. Approximately 1 mg BXO was eluted from the column. The binding capacity of the column was, therefore, approximately 1 mg BXO/5 mg immobilized polyclonal IgG.

3.3.2 Partial purification of BXO from bovine milk

A crude preparation (i.e. post-ammonium sulphate precipitation) of bovine enzyme from fresh milk was provided (J Khan). The sample, 0.5 ml with total XOR activity 3.5 ± 0.23 nmoles/minute/mg, was applied to the anti-(BXO) IgG/protein A/Sepharose 4B affinity column. It was slowly recycled through the gel for 8–12 hours at 4°C. The gel was washed with 10 mM phosphate buffer, pH 8.0, until the A_{280} trace had returned to baseline (i.e. zero non-specific protein elution).

Bound protein was eluted with 0.05 M diethylamine containing 0.5 M sodium chloride. The unadsorbed fraction was recycled through the washed matrix and a second bound peak was eluted. Eluted fractions were collected in 1/20 volume 1 M phosphate buffer, pH 6.8.

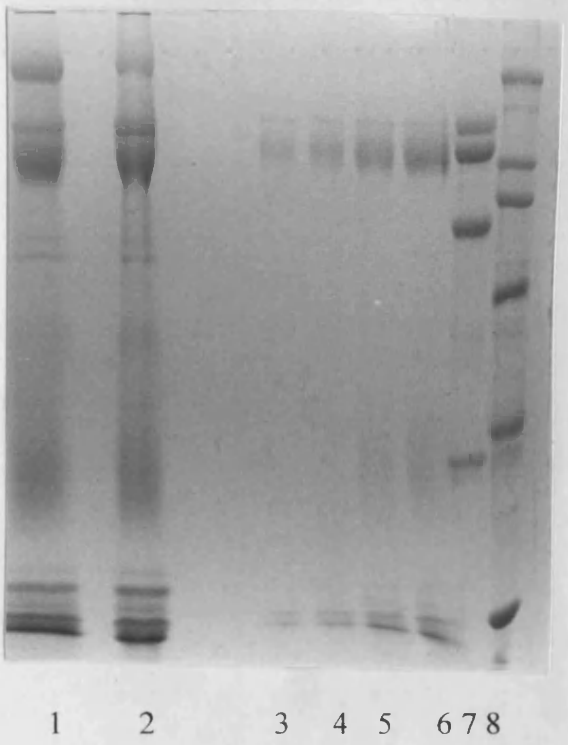
The peaks were pooled, concentrated, exchanged into PBS and assayed, spectrophotometrically, for total XOR activity (Materials and Methods 2.2.5). The spectrophotometric assay was repeated five times, and the total BXO activity of the eluted fraction was found to be 14.5 ± 1 nmoles/minute/mg. Thus, active bovine enzyme can be eluted from the column under the conditions described.

The experiment was repeated and the eluted bound fractions were pooled, concentrated and visualized by SDS-PAGE. The gel showed the bound protein with a double band at approximately 150 kDa, characteristic of the XOR, with a minor band at approximately 80 kDa.

3.3.3 Extraction of 'HXO-like' protein from human milk

The experiment described previously was repeated with a crude preparation of human milk (i.e. post-ammonium sulphate precipitation; D Powell). The sample (1.0 ml), was slowly recycled through the gel for 8–12 hours at 4°C. The gel was washed with 10 mM phosphate buffer, pH 8.0, until the A_{280} trace had returned to baseline (i.e. zero non-specific protein elution), and bound protein was eluted with 0.05 M diethylamine containing 0.5 M sodium chloride. Eluted fractions were collected in 1/20 volume 1 M phosphate buffer, pH 6.8.

The eluted bound fractions were pooled, concentrated, exchanged into PBS and visualized by SDS-PAGE (Photograph 7). There is no trace of 80 kDa contaminant in the eluted fractions, though a faint band corresponding to a molecular weight of approximately 40 kDa could be seen on the gel. The binding capacity of the column for partially purified HXO was approximately 0.15 mg (i.e. 15% binding capacity for BXO).



Photograph 7. SDS-PAGE gel showing partial purification of HXO from human milk using the anti-(BXO) affinity column.

1. Crude HXO
2. Unadsorbed fraction

3–6. Adsorbed fraction (at different loading concentrations)

7. BXO
8. Molecular weight markers

3.3.4 Partial purification of BXO from bovine liver

Fresh bovine liver was obtained from an abattoir and stored at -70°C, for 1–6 months. Samples were thawed at room temperature when needed.

A portion (12 g) was thawed at room temperature and roughly chopped in to small pieces. Tissue, 25% (w/v) in distilled water containing 4 mM dithiothreitol and protease inhibitors – leupeptin, antipain, aprotinin and pepstatin A (all at 1 μl/ml), was homogenized on ice. The crude homogenate was buffered with concentrated (x 5 normal PBS), 10% (v/v), and centrifuged at 35,000 *g* for 30 minutes at 4°C.

The pellet was discarded and finely ground ammonium sulphate was added to the supernatant at 20 g/100 ml. The suspension was stirred at 4°C for 10 minutes and then centrifuged at 15,000 *g* for 30 minutes at the same temperature. The pellet was discarded and further ammonium sulphate was added to bring the final concentration to 40 g/100 ml. The suspension was stirred and centrifuged as before.

The pellet was suspended in PBS containing DTT and protease inhibitors as described for the homogenization solution, and dialysed against several changes of this PBS over 24 hours. The dialysed supernatant was filtered through a series of disposable filter units, 1.2 μm to 0.22 μm (Millipore UK Ltd) and used immediately.

The anti-(BXO) IgG/protein A gel was equilibrated with PBS. The crude bovine liver preparation was buffered with 1/10 volume concentrated (x 5 normal) PBS.

The liver preparation was slowly recycled through the affinity column overnight. Unbound and weakly bound protein was washed from the gel with PBS and then PBS containing 0.5 M sodium chloride. Bound protein was eluted with 0.05 M diethylamine containing 0.5 M sodium chloride. Fractions were collected in tubes containing 1/20 volume concentrated (x5) PBS. Pooled fractions were concentrated, exchanged into PBS and assayed for total BXO activity (Materials and Methods 2.2.5; Table 13).

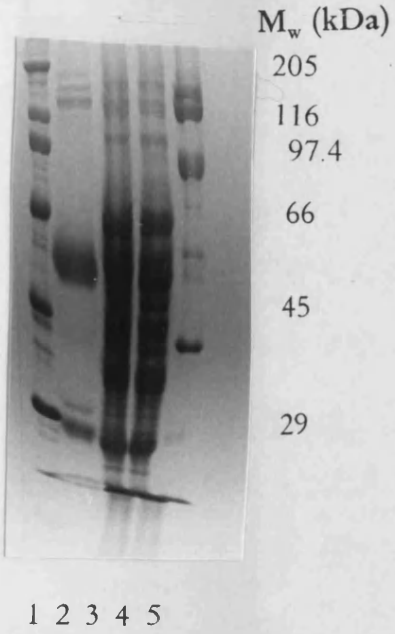
Fraction	Number of assays (n)	Mean BXO activity (nmoles/minute/mg)	% CV
Crude	2	1.25	7.5
Adsorbed	3	33.5	2.6
Unadsorbed	2	0.51	8.9

Table 13. Bovine liver fractions were assayed spectrophotometrically for BXO activity.

Samples of the crude, unadsorbed and bound fractions were run on SDS-gel (Photograph 8). A Western blot was performed on the bound fraction using the anti-(BXO) mAb, B5B4, and affinity-purified anti-(BXO) pAb (Photograph 9). For this blot, pAb was detected using an alkaline phosphatase system and mAb was detected using the HRP system. The results are summarized in Table 14.

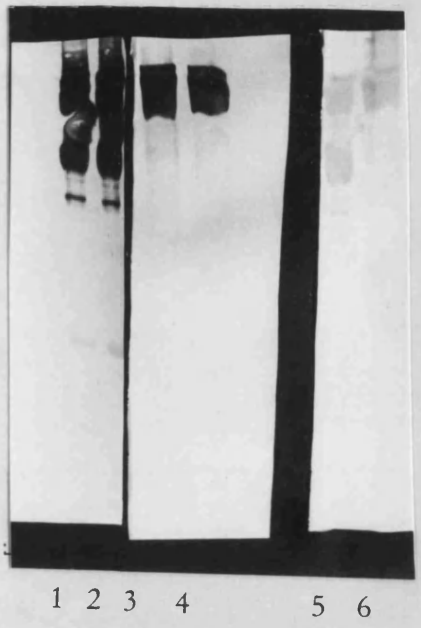
Both polyclonal and monoclonal antibody recognized bands corresponding to molecular weights 129–150 kDa, indicating that these bands are BXO protein. The molecular weights of bands running further down the gel suggest that these might be the heavy and light chains of polyclonal anti-(BXO) antibody stripped from the affinity column during elution. However, as the polyclonal anti-(BXO)

antibody in the Western blot was detected with goat anti-(rabbit) IgG, these bands would have been expected to stain darkly if this was the case. In fact, the band corresponding to 51–63 kDa showed only very light staining that is not consistent with this scenario.



Photograph 8. SDS-PAGE gel showing fractions from anti-(BXO) affinity purification using bovine liver homogenate.

1. Molecular weight markers
2. Adsorbed fraction
3. Crude homogenate
4. Unadsorbed fraction
5. BXO



Photograph 9. Corresponding Western blot using anti-(BXO) pAb at 30 μg/ml, and supernatant from the cell line B5B4.

1, 2. BXO (B5B4)

3, 4. Adsorbed fraction (B5B4)

5. BXO (pAb)

6. Adsorbed fraction (pAb)

Band	Detected by monoclonal antibody	Detected by polyclonal antibody
150 kDa	✓	✓
141 kDa	✓	✓
129 kDa	✓	✓
51–63 kDa	×	Faint
27 kDa	×	×
24 kDa	×	×

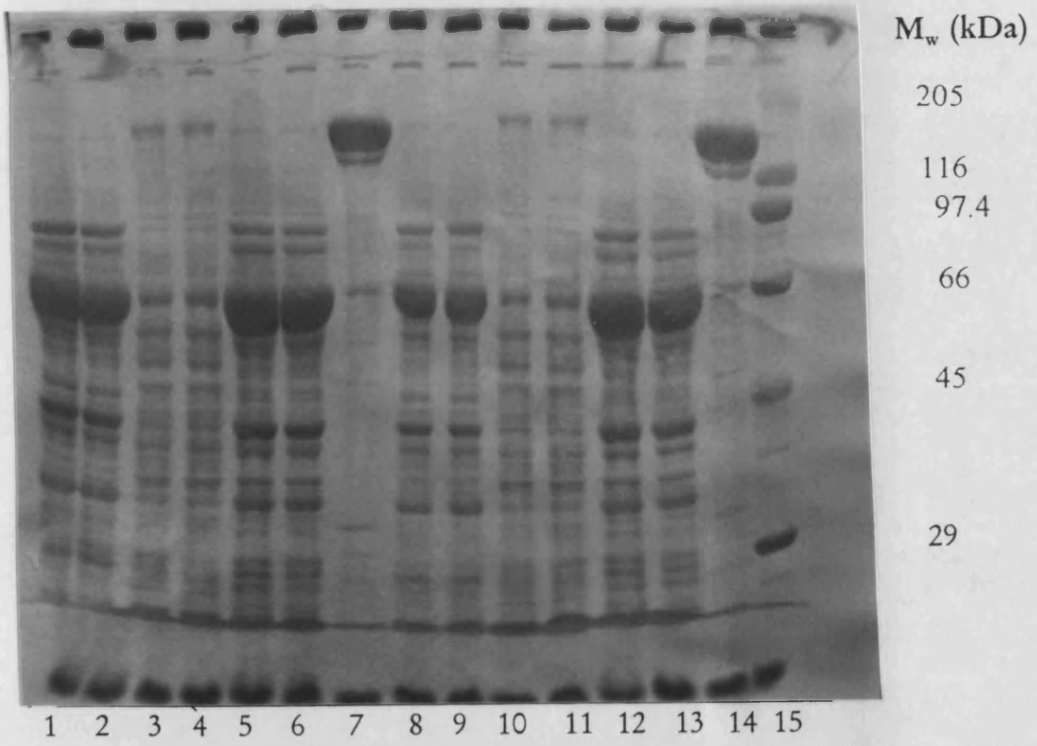
Table 14. Blotting the bound peak with monoclonal and polyclonal anti-(BXO) antibody showed similar patterns.

3.3.5 Visualization of 'HXO-like' protein in three human tissues

Homogenates of human smooth muscle, heart and liver (removed at post-mortem and deep frozen at -70°C for up to 6 months) were prepared as described previously (Results 3.6.4). Samples from each homogenate were subjected to SDS-PAGE followed by Western blotting using a multi-antibody approach (modified from Lee *et al.*, 1988).

After staining with Ponceau S [0.02% (v/v) in 3% trichloroacetic acid; Photograph 10], the blot was cut into two halves. One half of the blot was probed with the anti-(BXO) IgM mAb, B5B4, and the Vectastain (HRP) detection system (Photograph 11). The other half was probed with a mixture of affinity-purified anti-(BXO) pAb and the anti-(HXO) mAb, 2A3 (Photograph 12). The pAb was detected using HRP-conjugated, species-specific, goat anti-(rabbit) IgG and diaminobenzidine, which gave brown bands. The mAb was detected using alkaline-phosphatase-conjugated, species-specific, goat anti-(mouse) IgM and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma Fast™ BCIP/NBT buffered substrate tablets), which gave dark blue bands. Bands recognised by both antibodies were characterized by brown/black staining, which was not as dark as NBT only. It was, therefore, easy to distinguish between protein bands recognised by the different antibodies. As the coloured bands are not easy to distinguish from Photograph 12, the results of molecular weight estimation of bands recognised are presented in Tables 15–17.

In addition to probing homogenates immobilized on nitrocellulose, small samples of homogenate were passed through the anti-(BXO) affinity column as described previously (Results, 3.3.4). Adsorbed protein was eluted with 0.05 M diethylamine, pH 11.5, containing 0.5 M sodium chloride. The sequential elution traces for smooth muscle, heart and liver are presented (Figure 21). Protein peaks adsorbed by anti-(BXO) pAb, and eluted at pH 11.5, were observed for the three tissues. Heart fraction contained less protein and, hence, the elution profile differed from those of smooth muscle and liver.



Photograph 10. Ponceau S-stained nitrocellulose blotted against crude human smooth muscle, liver and heart homogenates on an SDS-PAGE gel.

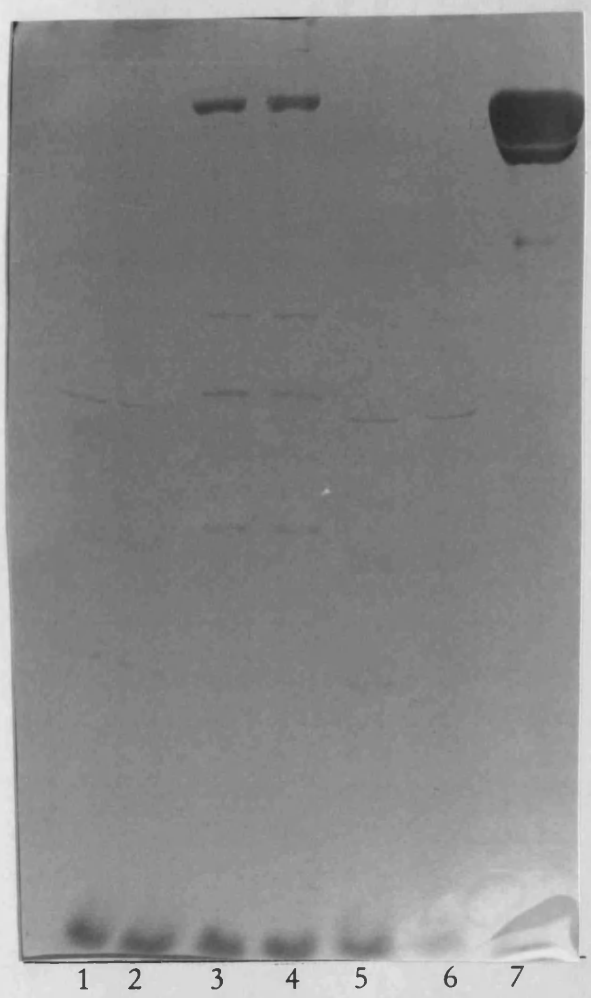
1, 2, 8, 9. Human smooth muscle

3, 4, 10, 11. Human heart

5, 6, 12, 13. Human liver

7, 14. HXO

15. Molecular weight markers



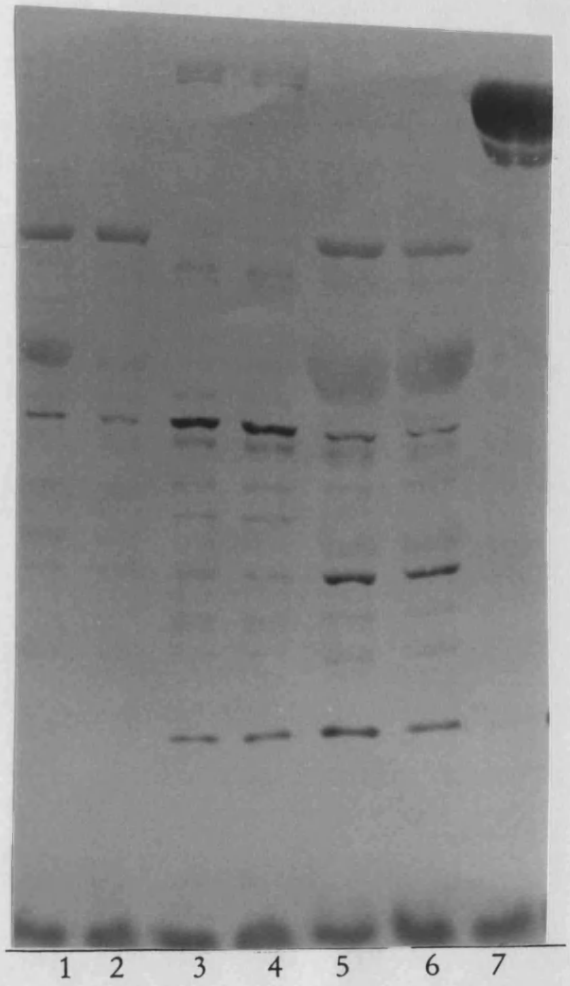
Photograph 11. Western blot using supernatant from the cell line B5B4 against crude human smooth muscle, liver and heart homogenates.

1, 2. Human smooth muscle

3, 4. Human heart

5, 6. Human liver

7. HXO



Photograph 12. Western blot using supernatant from the cell line 2A3, and affinity-purified anti-(BXO) pAb, against crude human smooth muscle, liver and heart homogenates.

1, 2. Human smooth muscle

3, 4. Human heart

5, 6. Human liver

7. HXO

Liver			
Estimated Mw (kDa)	**B5B4 detection**	**2A3 detection**	**Anti-(BXO) pAb detection**
91		+	+
63			+
58	+	+	
55			+
51			+
45			+
41	+	+	
39			+
33			+
28	+	+	

Table 15. Interpretation of the multi-antibody Western blot with crude human liver homogenate.

Smooth muscle			
Estimated Mw (kDa)	B5B4 detection	2A3 detection	Anti-(BXO) pAb detection
93		+	+
74			+
64			+
61			+
59	+	+	
45			+
42			+
28	+		

Table 16. Interpretation of the multi-antibody Western blot with crude human smooth muscle homogenate.

Heart			
Estimated Mw (kDa)	B5B4 detection	2A3 detection	Anti-(BXO) pAb detection
150	+		+
143		+	+
91	+		+
85			+
68	+		
60	+	+	
59			+
52			+
48			+
42			+
38			+
27		+	

Table 17. Interpretation of the multi-antibody Western blot with crude human heart homogenate.

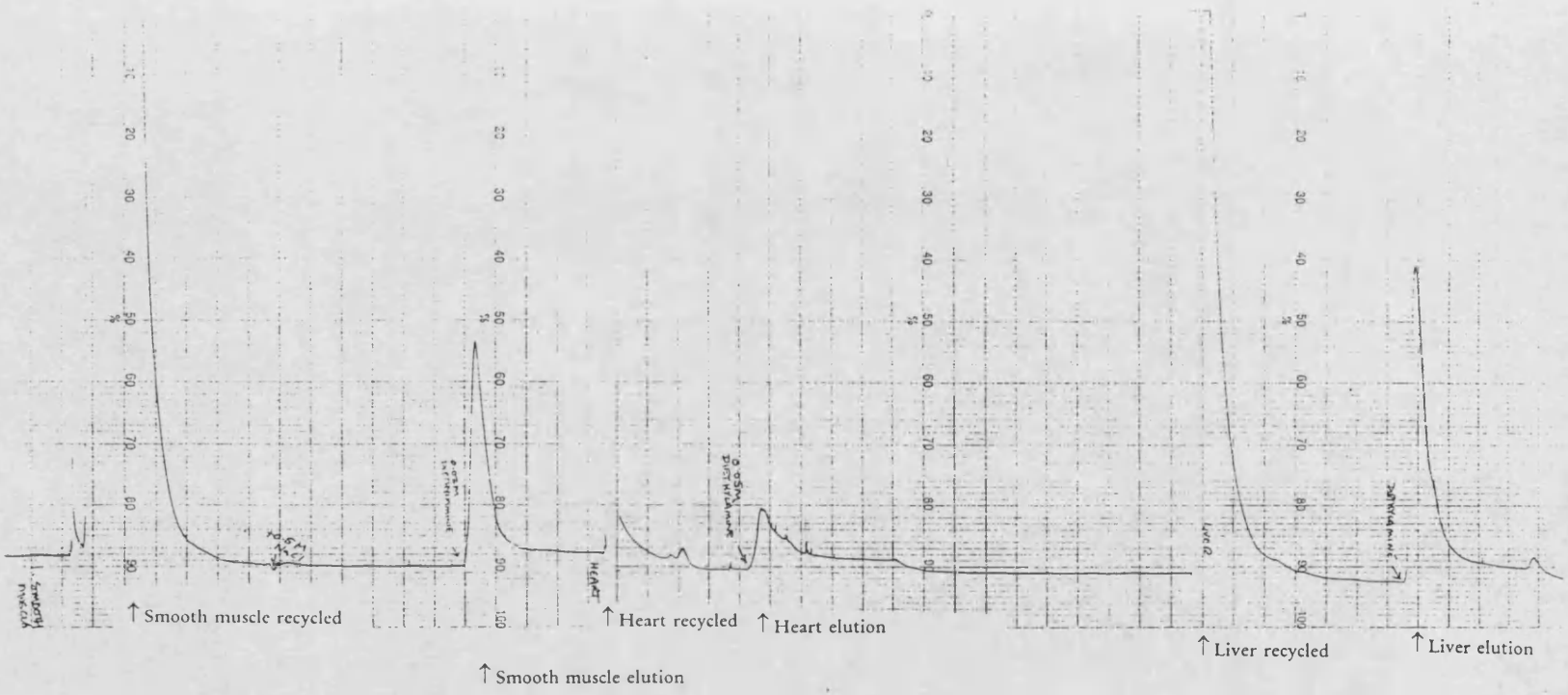


Figure 21. Elution profile of human smooth muscle, heart and liver from the anti-(BXO) affinity column, using 0.05 M diethylamine, pH 11.5, containing 0.5 M sodium chloride, as eluant.

3.3.6 Partial purification of 'HXO-like' protein from human liver

Samples of human liver, removed at post-mortem, were deep frozen at -70°C for up to 6 months.

A portion (wet weight 3.8 g) was removed and thawed at room temperature. Crude homogenate was prepared by blade homogenization, ammonium precipitation, centrifugation and pellet dialysis, as described previously (Results 3.6.4). The dialysed supernatant was filtered through a series of disposable filter units, 1.2 μm to 0.22 μm and used immediately.

The anti-(BXO) IgG/protein A gel was equilibrated with PBS. The crude human liver preparation was buffered with 1/10 volume concentrated (x 5 normal) PBS.

The liver preparation was slowly recycled through the affinity column overnight. Unbound and weakly bound protein was washed from the gel with PBS and then PBS containing 0.5 M sodium chloride. Bound protein was eluted with 0.05 M diethylamine containing 0.5 M sodium chloride; fractions were collected in tubes containing 1/20 volume x 5 PBS. Fractions containing bound protein were pooled, concentrated, exchanged into PBS and assayed for total HXO activity (Table 18).

Fraction	Total protein (mg)	Number of assays (n)	Total HXO activity (nmoles/minute/mg)	%CV
Crude	110	3	3.7	10.8
Adsorbed	0.40	3	6.8	18.7
Unadsorbed	56	4	13.0	21.2

Table 18. Crude human liver preparation was recycled through the anti-BXO/protein A affinity column. Fractions were assayed spectrophotometrically for total XOR activity.

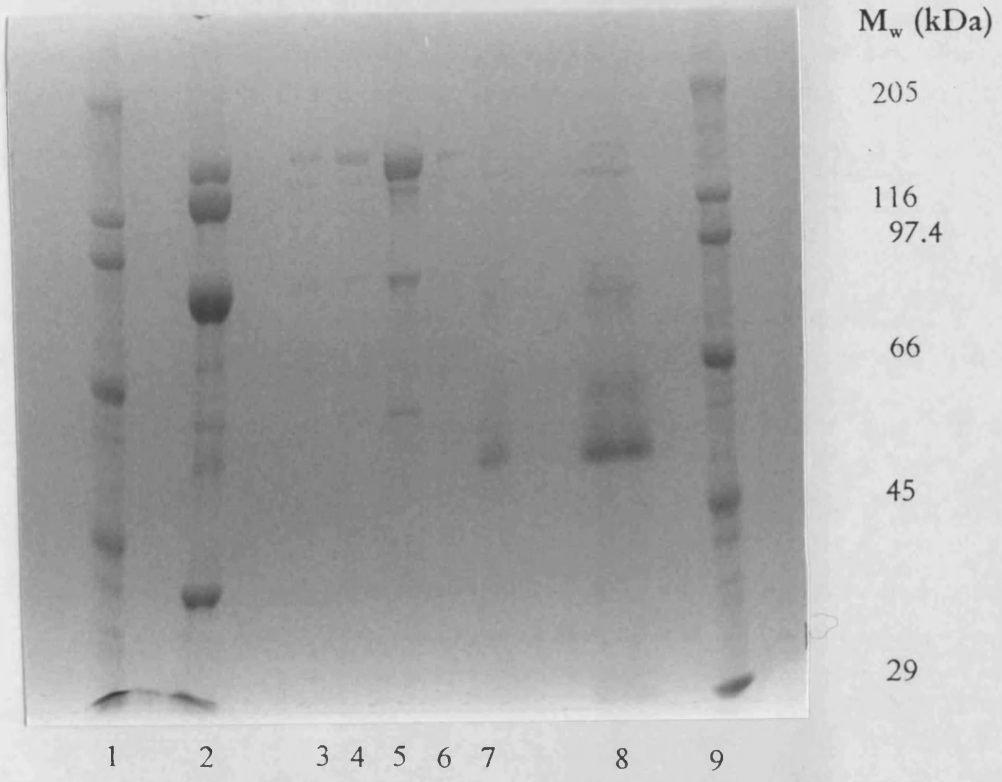
The reason for the seemingly nonsensical results for the unadsorbed fraction is unclear. While the protein content appears to have diminished by nearly 50%, the XOR activity is almost double that of the crude fraction.

Samples from each stage were run on SDS-PAGE (Photograph 13). The lane with bound protein (lane 8) showed bands corresponding to molecular weights 141 kDa, 129 kDa, 89 kDa, 65 kDa, 53 kDa.

Photograph 14 shows the greatly increased number of bands that occurred when protease inhibitors were omitted from the protocol.

As a comparison, human liver homogenate was passed through a heparin-agarose column similar to that used for purification of HXO from milk (Appendix II). Elution conditions were similar to those used for milk enzyme, with bound proteins eluted with stepwise increases in ionic strength of the eluent. In milk preparations, HXO elutes with 0.4 M sodium chloride and, indeed, a peak similar to HXO (in terms of estimated molecular weight) was seen

in the 0.4 M eluate (running with Mw=147 kDa; pure HXO runs with Mw=143 and 129 kDa on the same gel). The '129-kDa-like' band was not detectable in the 0.4 M eluate, but a similar double band pattern was seen in the crude lane (i.e. bands running at M_w=147 and 135 kDa). The four major bands eluting with 0.4 M sodium chloride corresponded to M_w=56, 50, 43 and 34 kDa. In addition, bands also occurred corresponding to molecular weights below 29 kDa (Photograph 15).

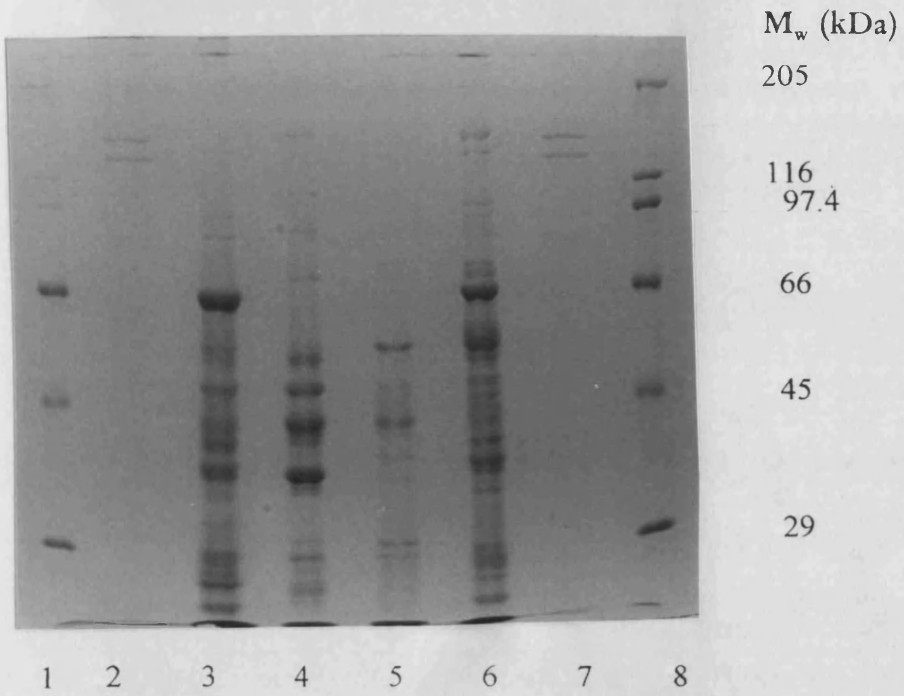


Photograph 13. SDS-PAGE gel showing anti-(BXO)-pAb-adsorbed fraction extracted from human liver homogenate.

1. Molecular weight markers
2. BXO
3–6. HXO
7, 8. Adsorbed fraction from human liver homogenate
9. Molecular weight markers

Photograph 14. SDS-PAGE gel showing anti-(BXO)-pAb-adsorbed fraction extracted from human liver homogenate in the absence of protease inhibitors.

1. Molecular weight markers
2. Unadsorbed fraction
3. Crude human liver homogenate
4. Adsorbed fraction
5. Crude fraction
6. HXO
7. BXO



Photograph 15. SDS-PAGE gel showing fractions obtained from human liver homogenate using heparin chromatography.

1. Molecular weight markers
2. HXO
3. Eluant = sodium phosphate buffer containing 1 M sodium chloride
4. Eluant = sodium phosphate buffer containing 0.4 M sodium chloride
5. Eluant = sodium phosphate buffer containing 0.1 M sodium chloride
6. Crude fraction
7. HXO
8. Molecular weight markers

3.3.7 Partial purification of 'HXO-like' protein from human heart

Two samples of post-mortem human heart were deep frozen at -70°C for 2 months, and then thawed at room temperature.

Crude homogenate was prepared by blade homogenization, ammonium precipitation, centrifugation and pellet dialysis, as for bovine liver (Results 3.6.4). The dialysed supernatant was filtered through a series of disposable filter units, 1.2 μm to 0.22 μm and used immediately.

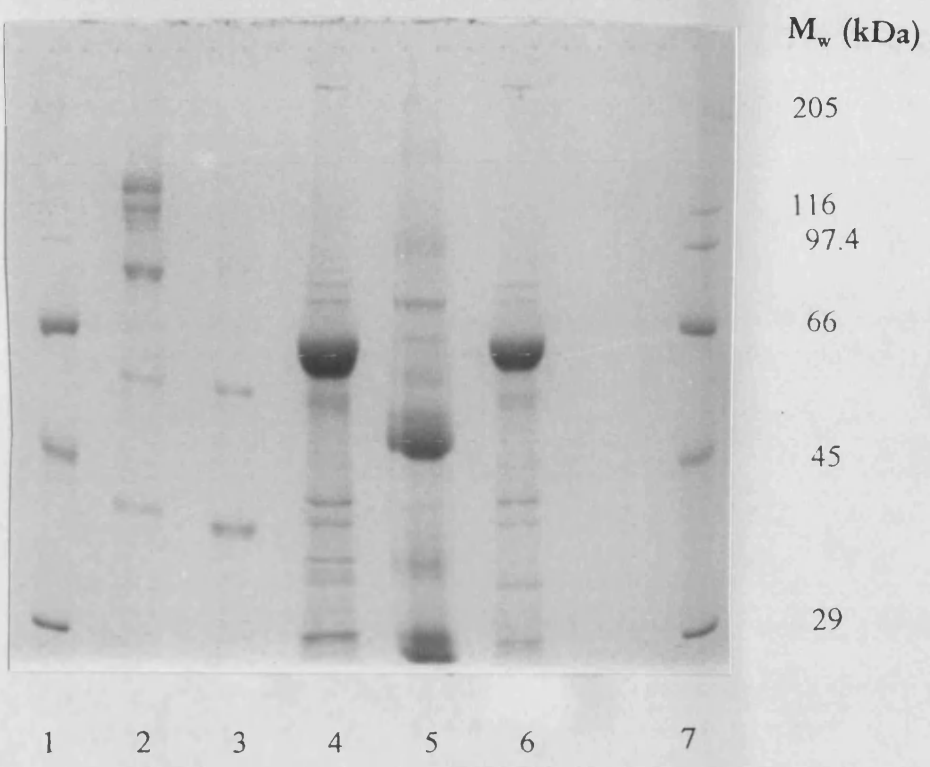
The anti-(BXO) IgG/protein A gel was equilibrated with PBS, and crude human heart preparation was buffered with 1/10 volume concentrated (x 5 normal) PBS.

The heart preparation was slowly recycled through the affinity column overnight at 4°C. Weakly bound protein was washed from the gel with PBS followed by PBS containing 0.5 M sodium chloride. Bound protein was eluted with 0.05 M diethylamine containing 0.5 M sodium chloride; fractions were collected in tubes containing 1/20 volume x 5 PBS.

Fractions containing bound protein were pooled, concentrated, exchanged into PBS. The experiment was repeated several times, and fractions were either assayed for HXO activity or subjected to SDS-PAGE and Western blotting. Because of the small amounts of protein obtained on elution with 0.05 M diethylamine and 0.5 M sodium chloride, it was not possible to assay the same enzyme peak for activity and visualize the proteins on SDS-PAGE.

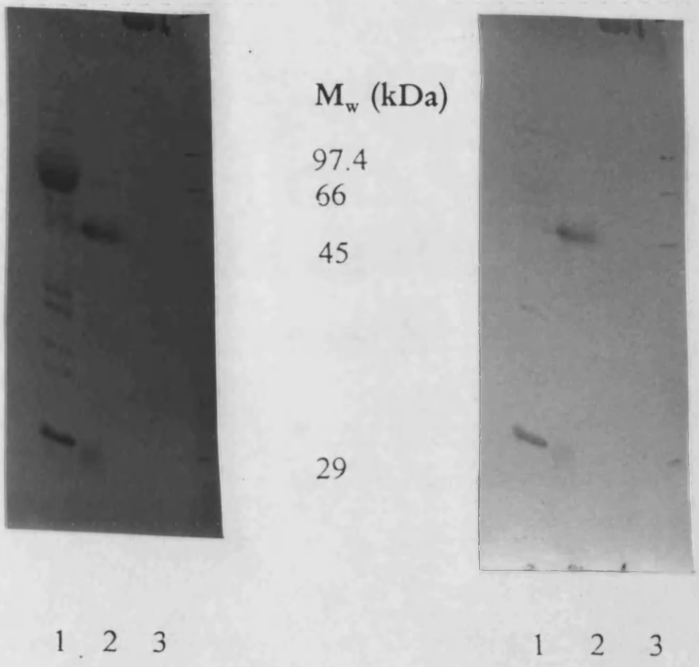
Photograph 16 shows the band pattern of protein eluted from the anti-(BXO) column with one sample of human heart homogenate. The bands correspond to the following molecular weights, expressed in kDa: 139 (faint

staining), 92, 70, 60, 47 (strong staining), 43, 27, < 27 (strong staining). Bands corresponding to the approximate position of the intact HXO monomer did not occur. Fractions obtained from another sample of human heart homogenate were probed with the anti-(HXO) mAb 2A3. On this occasion, the preceding electrophoresis was stopped at an earlier stage, so that bands corresponding to lower molecular weights could be visualized more easily. With Ponceau S (i.e. non-specific protein) staining, the bands corresponding to bound protein ran with approximate M_w 66 and 30 kDa (Photograph 17). The anti-(HXO) mAb recognised both the bands, though the 66-kDa-band appeared to be more strongly stained (Photograph 18).



Photograph 16. SDS-PAGE gel showing anti-(BXO)-pAb-adsorbed fraction extracted from human heart homogenate.

1. Molecular weight markers
2. BXO
3. HXO
4. Crude human heart homogenate
5. Adsorbed fraction
6. Unadsorbed fraction
7. Molecular weight markers



Photograph 17. Ponceau S-stained nitrocellulose blotted against human heart homogenate fractions shown on Photograph 16.

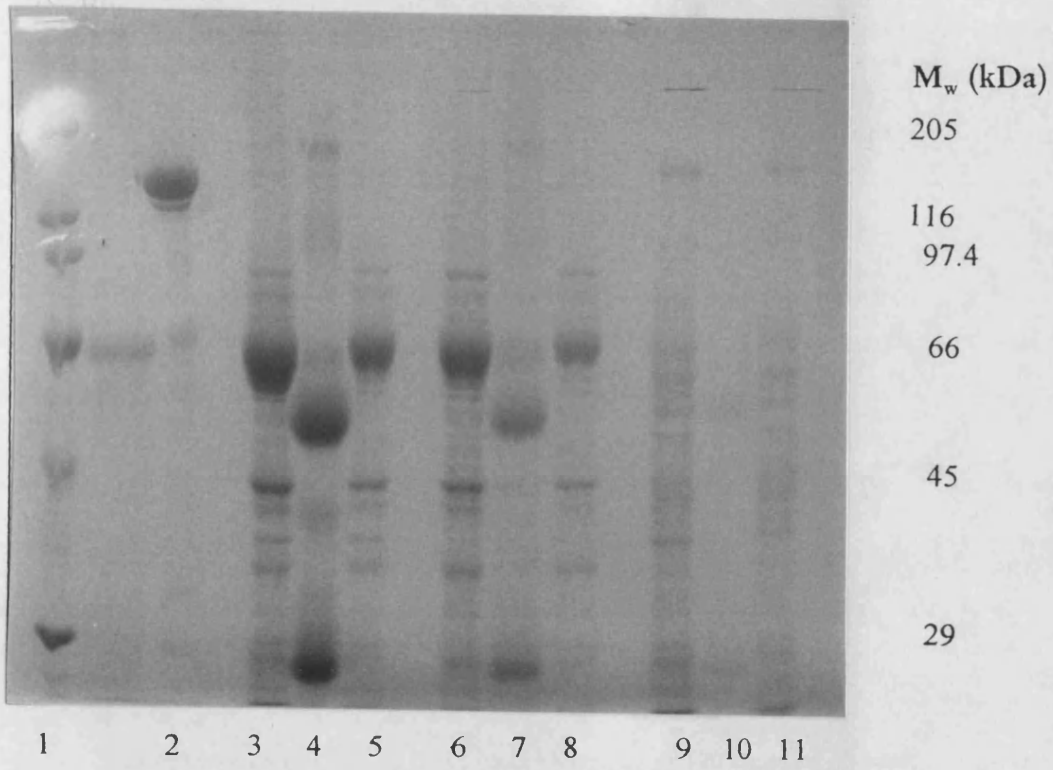
1. Crude human heart homogenate
2. Adsorbed fraction
3. HXO

Photograph 18. Western blot using supernatant from the cell line 2A3, against human heart homogenate fractions shown on Photograph 17.

1. Crude human heart homogenate
2. Adsorbed fraction
3. HXO

Fractions obtained from further human liver and heart preparations were run on the same SDS-polyacrylamide gel (Photograph 19). Bands corresponding to M_w 55 and < 29 kDa were evident for the adsorbed fractions of both liver and heart. The adsorbed fraction from heart had additional, faintly staining bands corresponding to 146, 106 and 39 kDa; the absence of these bands in the liver fraction, however, may simply reflect the lower amount of protein loaded onto the gel.

Total XOR and XO activity was measured using the fluorescent assay (Materials and Methods 2.2.6; Table 19). The addition of allopurinol to the assay mix inhibited the production of isoxanthopterin (Figure 22). The unadsorbed fractions had negligible XOR activity (Figure 23).



Photograph 19. SDS-PAGE gel showing anti-(BXO)-pAb-adsorbed fractions extracted from human heart and liver homogenates.

1. Molecular weight markers
2. HXO
3, 6. Crude human heart homogenate
4, 7. Adsorbed fraction from heart
5, 8. Unadsorbed fraction from heart
9. Crude human liver homogenate
10. Adsorbed fraction from liver
11. Unadsorbed fraction from liver

	Sample 1	Sample 2
Tissue wet weight (g)	25.5	10.3
Crude protein (mg)	798	372
Unadsorbed protein (mg)	680	359
Bound protein (mg)	2.26	4.05
Mean XOR activity of unadsorbed fraction	negligible	negligible
Mean XO activity of bound protein and %CV (pmoles/min/mg)	3.02 (n=2; CV=14%)	4.10 (n=9; CV=28%)
Mean XOR activity of bound protein and %CV (pmoles/min/mg)	3.9 (n=2, CV=36%)	5.30 (n=6; CV=22%)

Table 19. Two human heart preparations were recycled through the anti-BXO/protein A affinity column. The fractions were assayed fluorimetrically for XOR and XO activity.

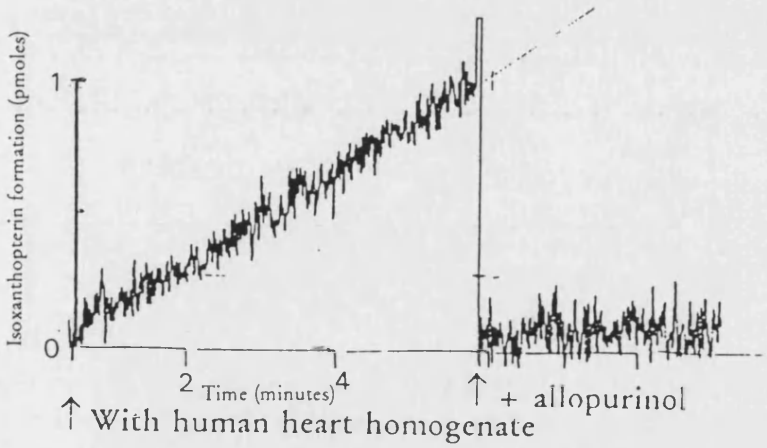


Figure 22. The effect of allopurinol on the rate of isoxanthopterin formation by an anti-(BXO) affinity-purified fraction from human heart homogenate.

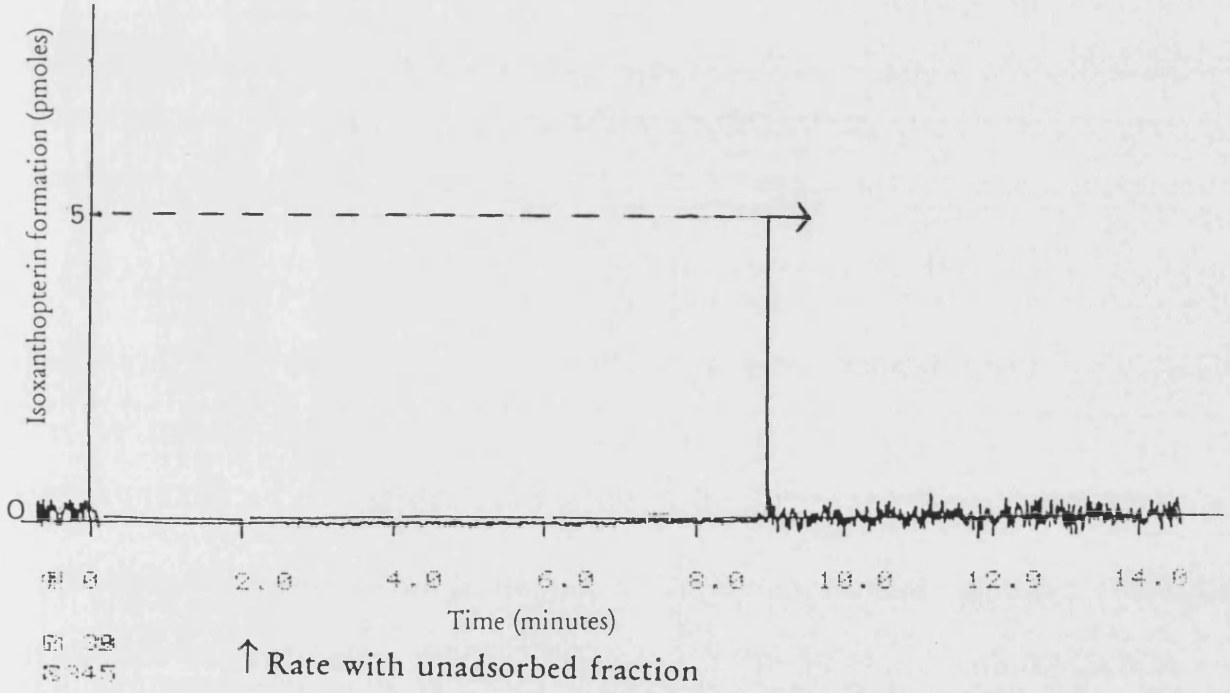


Figure 23. The unadsorbed fraction from anti-(BXO) affinity-purification of human heart homogenate had a negligible rate of isoxanthopterin formation.

3.3.8 XOR activity in rat tissue

In order to compare data from fluorimetric analysis of enzyme activity determined in-house with published data, samples of rat heart and rat liver were homogenized and assayed under the same conditions as the human tissue samples. Results are presented (Table 20).

Reproducibility was poor between the assays, and results appear to be significantly lower than those reported in the literature (see Discussion 4.7).

	Liver	Heart
Wet weight (g)	6.11	0.98
Homogenate protein (mg)	409	37
Mean XO activity and %CV (pmoles/minute/mg protein)	53.6 (n=5; CV=47%)	5.4 (n=3; CV=20%)
Mean XOR activity and %CV (pmoles/minute/mg protein)	69.2 (n=4; CV=32%)	7.6 (n=3; CV=15%)

Table 20. Samples of rat heart and liver were assayed for oxidase and total activity, using fluorimetric measurement of isoxanthopterin formation.

3.4 Rabbit aldehyde oxidase purification

Livers (52.5 g and 92.2 g) were obtained from 2 sacrificed rabbits. Crude homogenates were prepared as by ultracentrifugation and ammonium sulphate precipitation, and aldehyde oxidase was extracted (Materials and Methods 2.6.1). The final stage of the reported protocol (Warne and Stell, 1990) involved anion exchange, though in-house attempts to reproduce this stage were unsuccessful, and protein could not be eluted from the column.

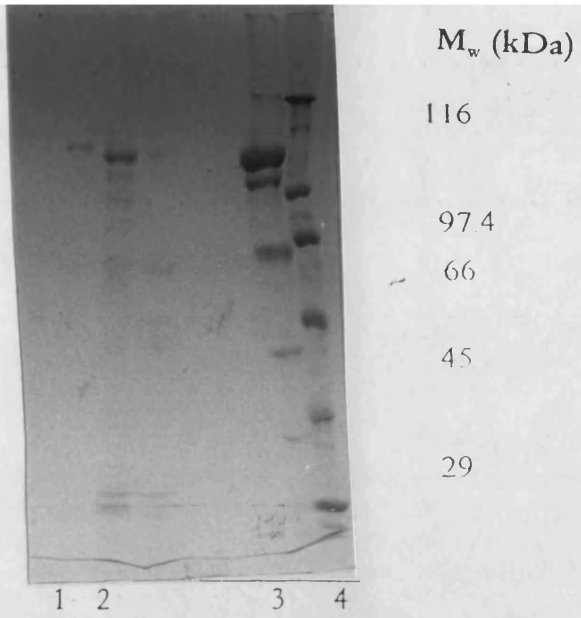
The pre-anion exchange fraction was analysed by SDS-PAGE and assayed for XOR and aldehyde oxidase activity (Table 21). The bound fractions from the *p*-aminobenzamidine Sepharose 6B column, and all subsequent fractions had negligible XOR activity. From SDS-PAGE, the rabbit aldehyde oxidase appears to run slightly more slowly than HXO, with an estimated molecular weight of 170 kDa compared with 158 kDa (Photograph 20).

The purification was repeated, with the gel filtration and anion exchange chromatography stages omitted (Photograph 21), and fractions were tested by BXO capture ELISA (Figure 24; Results 3.2.2.3). Crude and pooled peak fractions had negligible titre compared with BXO on ELISA.

Attempts by an undergraduate project student to purify bovine aldehyde oxidase using the same purification strategy were unsuccessful.

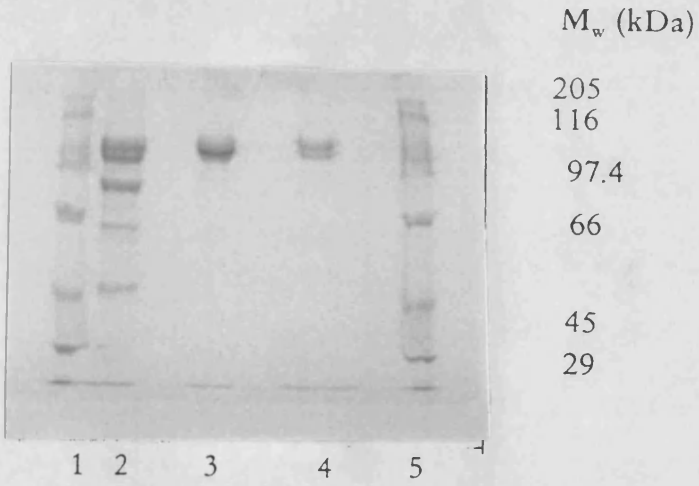
Fraction	Protein (mg/ml)	XOR specific activity (nmoles/minute/mg)	Aldehyde oxidase specific activity (nmoles/minute/mg)
Crude rabbit liver homogenate	7.25	1.0	37
Unadsorbed (post-benzamidine)	6.10	6.7	negligible
Benzamidine-adsorbed peak	0.15	4.8	105
Post-gel filtration	0.09	6.9	1450
HXO (purified in-house)	1.3	4.7	negligible
BXO (commercial)	8.1	4600	1.8

Table 21. Purified rabbit liver aldehyde oxidase was assayed for XOR and aldehyde oxidase activity. Commercially available BXO and freshly prepared HXO were also assayed.



Photograph 20. SDS-PAGE gel comparing aldehyde oxidase purified from rabbit liver with HXO and BXO.

1. Aldehyde oxidase (post gel filtration, pre-anion exchange chromatography)
2. HXO
3. BXO
4. Molecular weight markers



Photograph 21. SDS-PAGE mini-gel comparing aldehyde oxidase from rabbit liver with BXO and HXO.

1. Molecular weight markers
2. BXO
3. HXO
4. Aldehyde oxidase (post-*p*-aminobenzamidine chromatography)
5. Molecular weight markers

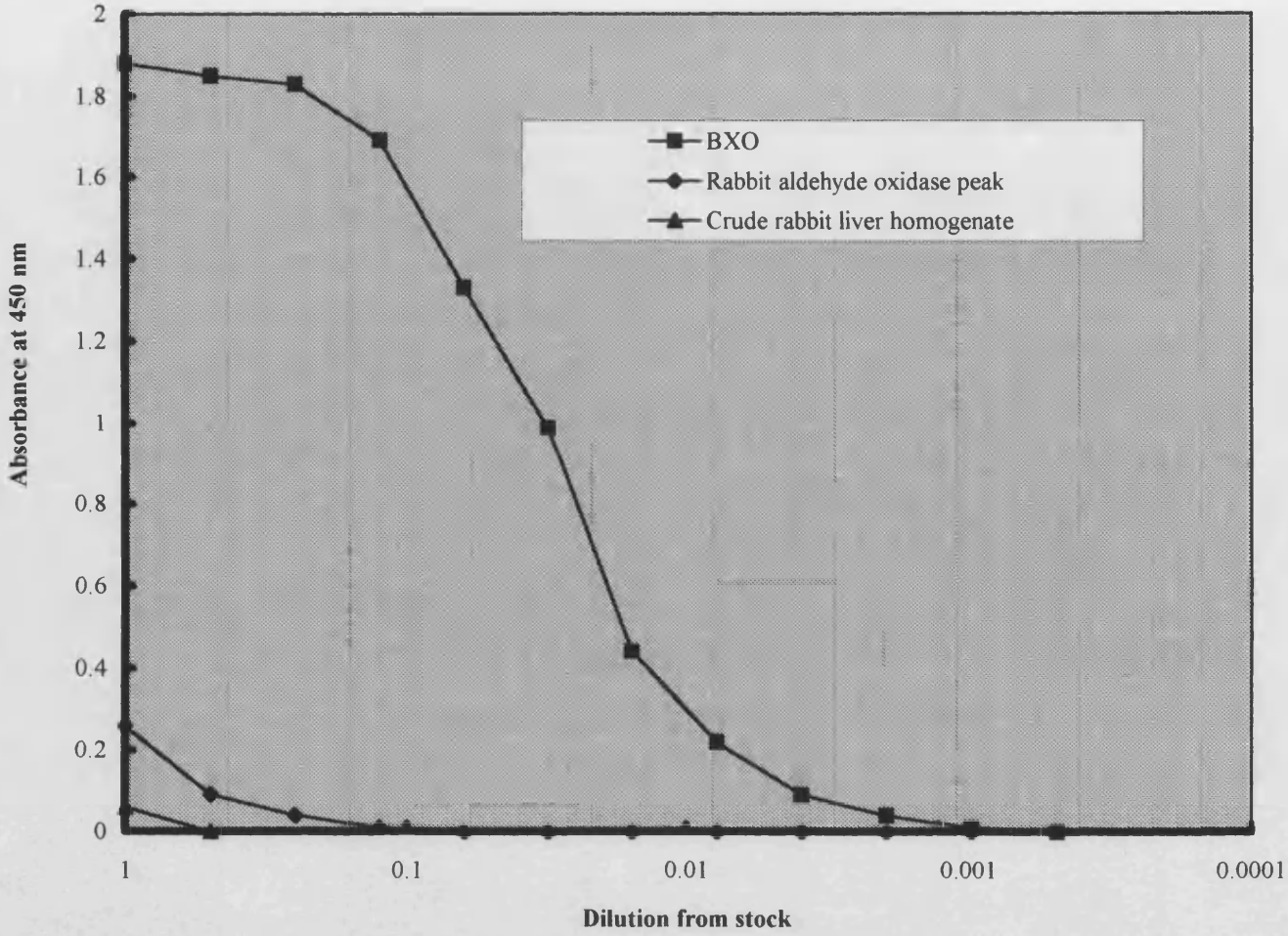


Figure 24. Titration curve for purified rabbit liver aldehyde oxidase, using the pAb/biotin-pAb anti-(BXO) capture ELISA.

4 Discussion

4.1 Aims of the project

The aims of the project were:

- to produce monoclonal and polyclonal anti-(XOR) antibodies
- to use these antibodies in a non-radioactive, semi-quantitative assay for XOR in tissue homogenates
- to investigate the use of anti-(XOR) antibodies in affinity purification of XOR from, particularly, human tissues.

4.2 Polyclonal antibody production

Rabbit polyclonal antiserum was produced routinely. Both BXO and HXO appeared to be highly immunogenic, and high titres were achieved after the first immunization.

The initial strategy for IgG isolation involved ammonium sulphate precipitation followed by ion-exchange chromatography. This was replaced by purification on a protein A-Sepharose 4B affinity gel, which was more convenient and gave a slightly higher yield of IgG (~ 100 mg/10 ml antiserum compared with 60–80 mg/10 ml; Results 3.1.1.1–2). A fraction of the IgG was further affinity-purified on a BXO- or HXO-Sepharose 4B affinity column. An IgG fraction and an affinity-purified fraction were prepared from each batch of antiserum and, when used together in ELISAs, care was taken to ensure that only antibodies from the same batch of antiserum were used.

The results from ELISA and Western blots of anti-(BXO) pAb against BXO and HXO indicate that avidity with bovine epitopes was greater than that with human epitopes, though the antibodies were cross-reactive. Although it is not surprising that the antibodies have the greatest avidity for the immunogen, it does indicate that some inter-enzyme epitope variation exists. The finding that the anti-(HXO) pAb have similar recognition of both the human and bovine enzyme forms, however, is unexpected. Either the more immunogenic HXO epitopes are conserved in the bovine enzyme or the properties of the affinity-purified anti-(HXO) antibodies reflect affinity-purification of selected paratopes.

The instability of HXO cross-linked to CNBr-inactivated Sepharose 4B was demonstrated, and made routine affinity-purification of anti-(HXO) antibodies impossible. Deterioration of the immobilized enzyme on the affinity matrix may have exposed only the more stable of the human epitopes. These more stable tertiary characteristics may also be well-conserved between HXO and BXO. Degradation would also, however, be expected to expose novel epitopes not exposed on the immobilized BXO molecules and, therefore, recognition of the HXO enzyme by the polyclonal population might be expected to be greater than that of BXO. By examining the SDS-PAGE gel of the breakdown products of HXO coupled to Sepharose 4B, it appears that the breakdown does not mirror the breakdown patterns of HXO seen on storage (Photographs 2 and 3). If the novel epitopes are not exposed in the test HXO sample, then these antibodies will not be detected and the avidity of the antiserum will be skewed.

Furthermore, that an additional elution stage (i.e. at low pH) was required for anti-(HXO) pAb using the HXO affinity column also indicates that the nature of some of the anti-(HXO) pAb/HXO interactions on the affinity column differed from those of the anti-(BXO) pAbs on the BXO affinity column.

4.3 Monoclonal antibody production

It was initially hoped to produce monoclonal hybridomas secreting anti-(XOR) IgG, as these are readily incorporated into ELISA and affinity chromatography. Accordingly, the failure to produce murine hybridoma cell lines secreting IgG was disappointing. It became important to use the antibodies available to develop techniques within the laboratory that could be optimised with anti-(XOR) IgG mAbs, should they become available.

The reason(s) why the established immunization schedule and subsequent fusion/clone protocol did not result in mouse IgG-secreting cell lines, is unclear. Historically, several other workers in our laboratory had experienced the same difficulties and, despite attempts to produce IgG-secreting hybridomas over 5 years, none had been obtained.

Clearly, in the immunization schedule used for the mice, the class-shift was not achieved and the secondary response also produced antibodies from the μ-class. It has been suggested, (D. Brennand; personal communication), that the IgM antibodies produced in the primary response may had such high affinity for the antigen that there was no maturation of class from μ to γ.

Another reason for the failure to provoke the classical secondary response would be if the secondary immunogen differed from the primary. If this was the

case, and common epitopes were not recognised, a second primary response would be provoked with stimulation of a different antigen-reactive lymphocyte. The enzyme preparation used as immunogen for each animal was stored at -20°C between immunizations. This was necessary because the enzyme purification protocol was being altered and improved constantly, and the purity of the final enzyme product was not constant. However, the intact enzyme is a relatively large immunogen and would certainly be partially proteolysed preceding antigen presentation. That breakdown on storage of the enzyme would differ from this partial proteolysis to the extent of a lack of common epitopes seems highly unlikely. In addition, the same problem occurred with BXO as immunogen. This is more stable than the human enzyme and is commercially available as a consistently pure form.

The final inconsistency was the ability of the same immunogen to provoke an IgG response in the rabbit, though the immune mechanism may be complicated by the existence of 'natural' anti-(XOR) antibodies. High concentrations of anti-(XOR) IgG have been reported in the sera of, among other animals, both the New Zealand white rabbit and the BALB/c mouse, (Bruder *et al.*, 1984). The positive titre of the normal (i.e. non-immunized) rabbit serum (Figure 9, Results 3.1.1) does indeed indicate circulating anti-(XOR) Ig is present in the laboratory rabbits used for pAb production, prior to immunization. In contrast, the normal mouse serum had negligible anti-(XOR) titre (Figure 13, Results 3.1.2), in disagreement with the findings of Bruder *et al.* (1984).

The proposed hypothesis to explain the presence of 'natural' anti-(XOR) is that XOR is released following cell damage and induces an immune response,

though the lack of immunological tolerance is not explained. Most 'natural' autoantibodies belong to the IgM class (Avrameas, 1983) and are low affinity and thymus-independent (reviewed in Roitt, 1994). If the rabbits used for pAb production were already producing anti-(XOR) before immunization, then the introduction of XOR at immunization would stimulate a secondary response. This may explain the relatively high titres after the first immunization. In mice, however, the first immunization provokes the primary response and XOR appears to act as a thymus-independent antigen, such that the response is mainly IgM and memory cells are not generated. However, this phenomenon does not appear to be common to all BALB/c mice, as several groups have reported the generation of hybridomas generating anti-(XOR) monoclonal IgG (Bruder *et al.*, 1983; Hellsten-Westing, 1993). The reported generation of mAbs in the same laboratory as that reporting naturally occurring anti-(XOR) antibodies (i.e. Bruder *et al.*, 1984) should be appreciated. If the mice used for hybridoma preparation already had circulating anti-(XOR) Ig, then a situation analogous to that described for rabbits might have occurred (i.e. response to immunization was secondary).

With hindsight, the inability of mice used in our laboratory to respond to XOR stimulation may have been signalled by the negligible anti-(XOR) titres of the normal mouse serum. In future, using another strain of mouse (e.g. C57Bl or CBA) or another species (e.g. rat) for anti-(XOR) hybridoma production might prove more successful. It would be of interest to investigate whether the existence of pre-existing anti-(XOR) antibodies impacts on the ability to generate IgG-secreting clones, and why XOR immunization in the mice used in our laboratory was not able to stimulate a secondary, IgG response.

Another strategy for the production of an IgG-secreting hybridoma line would be to isolate cells, if any existed, in which class switching had occurred. This technique requires a fluorescence-activated cell sorter (FACS) and a trained operator. Selection of small numbers of IgG-secreting cells followed by clonal expansion of the selected cells and a repetition of the exercise would be time-consuming and expensive, but represents an alternative approach to that used to date.

4.4 Anti-(XOR) monoclonal IgM

The anti-(XOR) hybridoma cell lines H3G7 and B5B4 appeared to recognise epitopes common to the bovine and human enzyme. Similarly, 2A3 and 2G3 gave positive staining on Western blots of both human and bovine enzyme.

That one of the established hybridoma cell lines, H2C2, was secreting IgG antibody against a contaminant illustrates the problem of enzyme purity for the purpose of antibody generation. This antibody did not stain nitrocellulose in a Western blot, and it was only when the antibody was coupled to Sepharose 4B

and used for affinity purification that the nature of the antigen was established. By comparing titres against different preparations of enzyme, the nature of the problem was confirmed (Figure 14, Results 3.1.3).

The IgM antibodies were of little use in ELISA. The absence of coloured end-product in the assay when one mAb population coated the plate and the alternative biotin-mAb population was used as the secondary detector antibody, would also suggest the two lines H3G7 and B5B4 recognised a common epitope. This was also indicated by subsequent surface plasmon resonance measurements (Appendix III).

Although the use of monoclonal IgM in ELISA was not pursued it is worth, for future reference, highlighting a potential problem associated with using hybridoma supernatant in a biotin-dependent assay. Biotin is present in the tissue culture media and, if not removed during antibody purification steps, will interfere with the assay. As high non-specific coloration did not routinely occur in the ELISAs using mAb, it appears that the biotin was either removed during antibody purification or was not immobilized on the plate. If future assays incorporate hybridoma supernatant, the effect of non-specific biotin on the results should be appreciated.

4.5 Development of ELISA to measure HXO and BXO

Inhibition ELISAs using the mAbs did not give useful reference curves, and optimization was not pursued. Capture ELISAs using biotin-labelled polyclonal IgG was, however, more successful.

The initial success of the ELISA could not be repeated using later batches

of antibodies, leading to speculation on differences between batches. From the results of later assays, it appears that the success of the first capture ELISAs was due to the non-affinity purified IgG fraction being free from enzyme. The failure to reproduce the preliminary results was confusing and much time was spent purifying further batches of IgG in order to replicate the initial standard curves. It was only when the majority of batches gave the pattern of high background coloration in the absence of antigen, consistent with the presence of immune complexes, that the magnitude of the problem was appreciated.

The presence of immune complexes of IgG and XOR in the antisera could, again, be explained by high concentrations of circulating anti-(XOR) Ig in normal rabbits (Discussion 4.2). The presence and distribution of immune complexes in blood reflects the amounts and relative proportions of antibody and antigen. It may be that later boosts of XOR resulted in antibody and antigen excess, with the consequential formation of circulating complexes.

It appears that the antibodies used in the first ELISAs were prepared from antiserum not containing immune complexes. It may have been collected after the first immunization, or it may be that the immunization schedule did not cause antibody and antigen excess in that rabbit. Later batches of antiserum, however, do appear to have contained immune complexes formed as a result of overstimulation.

Future immunization protocols should use minimum amounts of immunogen and continued boost/bleed regimens may not be possible. Immune complexes should be removed by precipitation or gel filtration.

Returning to the results achieved with the working capture ELISA, it was

initially surprising that substitution of HXO in the ELISA for BXO gave negligible end-point coloration. Given, however, that the anti-(BXO) pAb titre against HXO was an order of magnitude lower than that against BXO (Table 7, Results 3.1.1), in view of the sensitivity of the assay, this result seems to have reflected the lower anti-(HXO) affinity of the anti-(BXO) antibodies.

The use of biotin-labelled antibody in the capture ELISA, with subsequent detection by streptavidin- (or Extravidin-) labelled HRP, avoids the use of a tertiary anti-(Ig) antibody and thus avoids a possible source of background cross-reactivity. Biotinylation of antibody is straightforward; it is easy to manipulate the reaction to increase or decrease the amount of biotin label, as required. Since avidin has four biotin binding sites, it would be possible to amplify the assay by pre-incubating the streptavidin or Extravidin with biotin before incubation with the assay mixture. This was not investigated in this project but may merit future consideration.

In order to further improve the capture ELISA, particularly with regard to specificity, it will be necessary to generate two mAb populations, each recognising different epitopes. Even one anti-(XOR) monoclonal IgG, used with a polyclonal preparation, will be more powerful than the pAb/pAb ELISA. However, when one or more hybridomas secreting high-affinity monoclonal IgG becomes available, it should be possible to substitute mAbs for pAbs.

4.6 Partial purification of BXO

Preliminary experiments were designed using affinity-purified rabbit anti-(BXO) pAb cross-linked to protein A/Sepharose 4B.

Enzyme activity was totally removed from a crude bovine milk fraction by recycling through the anti-(BXO) affinity column (Results 3.3.2). This was an encouraging result as it demonstrated that the elution conditions (i.e. 0.05 M diethylamine, pH 11.5, containing 0.5 M sodium chloride) did not affect enzyme activity. However, it should be noted that the bovine milk sample had lower activity than that reported by Abadeh *et al.* (1992), 11.2 mU/ml compared with 73 mU/ml. The reason for this is unclear but the purpose of the experiment was simply to establish whether enzyme could be removed from a heterogeneous medium and whether the enzyme retained activity following elution from the affinity column. The lower capacity of the column for HXO (i.e. ~15%) is likely to be another consequence of the lower HXO affinity of anti-(BXO) antibodies.

This experiment was followed by recycling a crude bovine liver homogenate through the affinity column. Again, the purpose of this experiment was to test whether the affinity matrix could remove active enzyme from a crude tissue homogenate. Probing with the anti-(BXO) mAb B5B4 confirmed the presence of BXO in the eluted peak.

A concern with this method of affinity purification was that the relatively harsh elution conditions might strip the antibody from the column. Hence, it was important to establish that the eluted bands running with M_w around 25 and 50 kDa did not represent IgG heavy and light chains. Evidence supporting this was provided by the Western blot. If rabbit IgG was present in the adsorbed

fraction, when the nitrocellulose was incubated with the secondary antibody, the goat anti-(rabbit) antibody would have detected the corresponding heavy and light chains, and these bands would have stained heavily. This did not occur, indicating that rabbit antibodies were not present in the adsorbed fraction.

4.7 'HXO-like' protein in three human tissues

Protein recognised by anti-(XOR) mAbs and pAb was detected in human heart, smooth muscle and liver homogenates (Photographs 11 and 12; Results 3.3.5). From SDS-PAGE, it appears that antigenic bands running in the region of the intact HXO monomer occur only in heart, though even here the bands appear to correspond to slightly higher molecular weights (150 and 143 kDa compared with 141 and 136 kDa). Interestingly, the non-specific Ponceau S staining of nitrocellulose before probing with antibodies indicates faint bands running in the same position as the 141-kDa HXO band. These were not, however, recognized by either mAb or the pAb.

Similar patterns of detection occurred with smooth muscle and liver, though staining of bands corresponding to $M_w < 60$ kDa was weak with smooth muscle. The heart pattern differed from the smooth muscle/liver pattern above around 60 kDa, but was similar at lower M_w. If these bands represent HXO, this suggests differences exist between the cardiac and non-cardiac enzymes.

The recognition of major bands in the smooth muscle and liver lanes, corresponding to $M_w \sim 90$ and 70 kDa, by anti-(BXO) pAb and anti-(HXO) mAb, but not by anti-(BXO) mAb suggests that the epitope for B5B4 is not accessible

on these bands. That these bands do not occur in the heart lane further supports the hypothesis that the cardiac and non-cardiac forms differ. Obviously, cross-reactivity of the antibodies may mean that some or none of the bands correspond to HXO. Multi-antibody detection does, however, strengthen the evidence that certain bands indicate HXO protein.

4.8 Partial purification of 'HXO-like' protein from human tissue

The major limitation of this set of experiments was the use of an anti-(BXO) pAb affinity column to purify human enzyme. As discussed previously, the anti-(BXO) pAbs appeared to have lower affinity for the HXO. However, in the absence of enough affinity purified anti-(HXO) antibodies to construct a similar anti-(HXO) column, preliminary purifications were attempted with the anti-(BXO) column.

Results from an experiment using the column to remove HXO from a crude human liver preparation are confusing. A very small fraction of enzyme activity was removed but the unadsorbed fraction, despite having protein content approximately half that of the initial crude fraction, had almost double the activity of the crude. This implies that the activity of the unadsorbed fraction has been increased by the removal of protein. A high-molecular-weight, competitive inhibitor of XOR has been reported to copurify with XOR from bovine milk (Bray, 1959). Although non-dialysable, it is claimed that the effects of the inhibitor on enzyme activity may be overcome by precipitation with 60%

ammonium sulphate (Lee, 1973), or by high substrate concentration (~500 μM xanthine; Bray, 1959). In addition, Terada (1994) demonstrated the presence of a high-molecular-weight inhibitor in rabbit heart homogenate. If a similar inhibitor was present in the human liver preparation, and was adsorbed by the anti-(BXO) affinity column, the unadsorbed fraction would be expected to have reduced protein content and increased activity. Although this is an attractive theory, it means that anti-HXO inhibitor antibodies were generated by BXO immunization and purified with anti-(BXO) antibodies on affinity purification. As the commercially prepared BXO used to immunize rabbits showed no trace of high-molecular-weight contaminants on SDS-PAGE, and had high XOR activity, it is highly unlikely that the rabbit generated specific anti-inhibitor antibodies following immunization with BXO. Alternatively, *in vivo* association of native inhibitor with immunogen, might provoke an immune response.

If the presence of inhibitor somehow facilitated HXO recognition by the anti-(BXO) pAb, then enzyme associated with inhibitor would be expected to bind preferentially. On diethylamine elution, the enzyme/inhibitor complex would elute from the column. If the inhibitor had a molecular weight in the order of that indicated for the rabbit myocardial XOR inhibitor (i.e. approximately 370 kDa) it would not appear on SDS-PAGE gels, though fragments might be visualized. Furthermore, the presence of an inhibitor in the adsorbed fraction might explain the low XOR activity of this fraction, which is at odds with the amount of HXO protein seen on the SDS-PAGE gel. Preliminary evidence suggests that inhibitory activity might be associated with a 60-kDa fragment visualized on SDS-PAGE (see later discussion).

Another explanation of the increased HXO activity of the unadsorbed liver fraction could be the failure to totally remove BXO from the column following the bovine tissue experiments. The residual enzyme could be non-specifically washed out with the unadsorbed human homogenate. However, this seems unlikely as the column was thoroughly cleaned following the bovine experiments and baseline resolution was achieved with washes prior to the application of human homogenate. However, the possibility of bovine enzyme contamination cannot be disregarded. When the experiment was repeated later, after several other partial purifications, the protein concentration of the adsorbed peak in terms of initial tissue mass had decreased from 0.17 mg/g wet weight human liver to 0.0025 mg/g wet weight. This also suggests contamination in the first adsorbed peak, but could equally be explained by a decrease in efficiency of the column due to non-specific protein binding to the column.

As suggested by previous SDS-PAGE and subsequent Ponceau S staining of the blotted nitrocellulose (Photograph 10, Results 3.3.5), bands running in the same position as HXO were visualized in the crude liver homogenate (Results 3.3.6, Photograph 13). In contrast to the detection pattern achieved using anti-(BXO) pAb on crude homogenate, protein corresponding to these bands on SDS-PAGE was present in the anti-(BXO) pAb adsorbed fraction. The failure to detect the double band on Western blot may reflect low avidity for the 'HXO-like' band. Alternatively, the 'HXO-like' protein adsorbed by the affinity column may have been strongly associated with another protein (e.g. corresponding to bands ~ 64 and 27 kDa) for which the pAb has strong avidity. It may, therefore, have been non-specifically bound to the affinity column via strong association

with a specifically bound protein. It is tempting to suggest that these bands represent fragments of the inhibitor protein discussed earlier. The major band at around 64 kDa was recognised only by the pAb (Photograph 12, Results 3.3.5). If a subset of the anti-(XOR) pAb had been raised against this protein, had not been removed by anti-(BXO) affinity chromatography, and had, together with anti-(BXO) pAb, been cross-linked to Sepharose 4B, inhibitor may be present in the adsorbed fraction.

A similar band, running at around 64 kDa, was adsorbed from the bovine liver homogenate (Photograph 9, Results 3.3.9). In contrast to the human liver blot, probing of the bovine liver blot with anti-(BXO) pAb resulted in very faint staining of this band (Photograph 9, Results 3.3.9). Extending the hypothesis that this band corresponds to the presence of an inhibitory protein, that it is present in liver and smooth muscle extracts, but not heart, implies that this protein-mediated inhibition does not occur in heart. Although a preliminary finding, the indication that the affinity-chromatography unadsorbed liver fraction had higher specific XOR activity than the crude homogenate and that this was not the case for heart homogenate, also supports this theory.

In order to gain insight into another of the original project aims (i.e. to demonstrate the existence of the enzyme protein in human heart tissue) the experiment was repeated with human heart homogenate.

The experiment was repeated twice, under the same conditions, and the enzyme activities of the adsorbed fractions, measured using the fluorimetric pterin assay, were not significantly different (i.e. within 2 standard deviations).

In contrast to the situation with liver, the unadsorbed fraction had negligible XOR activity.

The values obtained for the XOR activity from extracts of human heart are considerably lower than the positive values reported, i.e. 0.27–1.61 μU/g wet weight of tissue compared with 0.3–37 mU/g wet weight of tissue (Muxfeldt and Schaper, 1987; Wajner and Harkness, 1989; de Jong *et al.*, 1990; Table 5, Introduction 1.2.4). It should be noted, however, that when enzyme activity has been recorded in human heart tissue previously, the tissue had been stored at 0–4°C and was, by implication, relatively fresh (Table 22). When tissue has been deep frozen, no activity has been detected. It is possible, therefore, that freezing decreases the activity to such a low level (i.e. in the range of pmoles/min/g tissue) that previous groups have not been able to detect it. However, Eddy *et al.* (1987) used a fluorimetric assay and conditions similar to those used in our laboratory, and did not detect enzyme in tissue that had been deep frozen. This group concluded that activity must have been below 2.0 nU/g. Immunohistochemical studies do not appear to be affected by storage, as XOR epitopes were detected in tissue stored at 0–4°C and -70°C. Thus deep-frozen tissue appears to maintain native epitope conformation sufficient for antibody recognition.

The choice of postmortem *vs* biopsy tissue sample may also affect assay results. Loss of up to 38% enzyme activity has been reported in postmortem rat liver for 3 hours at 22°C and then 2 hours at 4°C (Kooij *et al.*, 1992b). It appears, therefore, that XOR activity is affected by length and temperature of storage.

Author	XOR activity	Storage temperature
de Jong *et al.*, 1990	+	0–4°C
Eddy *et al.*, 1987	-	-70°C
Grum *et al.*, 1989	-	-80°C
Kooij *et al.*, 1992	-	-80°C
Muxfeldt and Schaper, 1987	+	0–4°C
Podzuweit *et al.*, 1991	-	-196°C
Wajner and Harkness, 1989	+	0–4°C

Table 22. The relationship between tissue storage temperature and reported XOR activity in human heart.

In both samples of heart protein eluted from the anti-(BXO) affinity column, XDH activity accounted for approximately 20% of total XOR activity. This differs from results reported by Wajner and Harkness (1987), and Muxfeldt and Schaper (1989). Both groups demonstrated that XDH contributed around 95% of XOR activity. Though care was taken to ensure that protease inhibitors and DTT were added at all stages of tissue homogenization, they may have been

removed from the bound fraction during affinity chromatography, allowing conversion to occur. Alternatively, the ratio of XDH:XO may reflect the enzyme state within the postmortem samples.

That the ratio is similar to that found in the rat tissues assayed, however, indicates that the homogenization conditions do not prevent all XDH to XO conversion, and approximately 80% of the enzyme converts to the oxidase form.

Reference to the results of XO and XOR activity fluorimetric assays of rat heart and liver indicates that the in-house fluorimetric assay may have been giving low values. Although the assay was calibrated with a fresh isoxanthopterin standard on each occasion, activities determined for rat heart and liver were lower than those reported. For example, Battelli *et al.* (1972) report that rat liver homogenate has XOR activity of 340 mU/g tissue, whereas our in-house assay gave a value of 4.6 mU/g. Similarly, reported values for XOR activity in rat heart range from 9 to 77 mU/g wet weight tissue (Table 1, Introduction 1.2.1) whereas the in-house assay measured 0.28 mU/g. As all in-house measurements were made using the same assay conditions and equipment, comparison between data sets can be made with confidence.

In practice, many factors are likely to affect XOR activity, including:

- gender and diet of laboratory animal
- tissue state (i.e. fresh *vs* frozen)
- method of extract preparation
- assay
- storage conditions (discussed previously)
- pathophysiological state of the tissue.

Though Eddy *et al.* (1987) used a fluorimetric assay similar to the in-house assay, homogenization conditions differed and, perhaps more significantly, they obtained heart from an unconscious rat, whereas the measurements reported here were made using heart removed shortly after death.

It may also be significant that, when a relatively high XOR activity has been reported for human heart, the assay has included a relatively high substrate concentration, 69–345 mM xanthine (Muxfeldt and Schaper, 1987) and 750 mM xanthine (Wajner and Harkness, 1989). The apparent K_m of the enzyme for xanthine is about 18 mM (Abadeh *et al.*, 1992) and other groups employing biochemical assays of human heart XOR activity have used xanthine concentrations in the range 50–95 mM (Eddy *et al.*, 1987; Podzuweit *et al.*, 1991). The presence of a high molecular weight XOR inhibitor, the effects of which can, reportedly, be overcome at high substrate concentrations has been described previously. The in-house spectrophotometric assay used 100 mM xanthine, which may have been too low to overcome the effect of similar inhibitors present in the homogenate. A further complicating factor is that tissue homogenates contain a

range of potential biochemical inhibitors of XOR activity, including uric acid, xanthine and NADH (Kaminski and Jezewska, 1979).

The pathophysiological state of the tissue may also affect the XOR activity, as enzyme leakage from diseased tissue is a well-known phenomenon. For example, XOR leakage from diseased liver has been reported (Shamma'a, 1965) leading to speculation that the reported XOR activity of human tissues removed from subjects with diseased liver, may be falsely high (Kooij *et al.*, 1992b). The presence of serum XOR should be borne in mind when assessing all reports of tissue levels. All work on human tissue has, to date, used samples obtained during surgery or postmortems, with no reference to the state of general health or, in particular, hepatic function. There is, therefore, no way of retrospectively relating XOR activity to evidence of hepatic disease. The use of immunohistochemical and molecular techniques will help overcome this problem, by localizing the enzyme directly. However, the source of XOR activity in tissue homogenates should be investigated fully (e.g. serum XOR measurements should be made in parallel).

Affinity-purification using the anti-(BXO)/protein A column was repeated several times in order to run the fractions on SDS-PAGE and Western blot. From the Western blot, it appears that the enzyme is present in human smooth muscle, heart, and liver. Detailed characterization of the enzyme from each source was not performed as the purification were extremely crude.

The adsorbed fraction can be termed 'HXO-like' as it was recognised by cross reactive anti-(BXO) Ig and eluted under conditions of high pH. This may, however, reflect the nature of the immobilized pAb, rather than *in vivo* HXO.

4.9 Aldehyde oxidase from rabbit tissue

Aldehyde oxidase is closely related to XOR and is reported to have an equally wide distribution (Krenitsky *et al.*, 1974). It is always a matter of concern, therefore, that the enzyme under investigation is neither wholly aldehyde oxidase, nor a preparation contaminated with that enzyme.

Purification of aldehyde oxidase from rabbit liver, using the method described (Methods 2.6.1), proved relatively simple. Although direct comparison of rabbit aldehyde oxidase with BXO or HXO was limited by consideration of interspecific variation, it was important to assess the specificity of the spectrophotometric and fluorimetric assays of enzyme activity. Rabbit aldehyde oxidase, when substituted for XOR in both the fluorimetric and spectrophotometric assay, had negligible activity. Similarly, BXO and HXO had negligible activity when substituted for aldehyde oxidase in the spectrophotometric assay for aldehyde oxidase activity. While this result was reassuring, an ideal comparison would have used aldehyde oxidase isolated from bovine and human tissue. Similarly, that anti-(BXO) pAb did not appear to cross-react with rabbit aldehyde oxidase on ELISA is not, in itself, an indication the antibodies raised do not cross-react with bovine aldehyde oxidase. One cause for concern is, however, that rabbit aldehyde oxidase runs slightly more slowly than HXO on SDS-PAGE (Photograph 20; Results 3.4), and as does the 'HXO-like'

protein extracted from human heart (Photographs 11 and 12; Results 3.3.5). Future experiments should address this issue, and a suggested approach is discussed later (Discussion 4.9.3).

The failure to obtain bovine aldehyde oxidase from liver using the same protocol as that used for rabbit enzyme may reflect the difference in reported aldehyde oxidase activities (i.e. rabbit aldehyde oxidase reduces 1980 nmoles ferricyanide/minute, contrasting with 100 nmoles ferricyanide/minute reported for Hereford cow; Krenitsky *et al.*, 1974). While the XOR plus aldehyde oxidase content of liver appears to be constant between the two species (i.e. approximately 160 mg protein/g tissue), the ratio of XOR:aldehyde oxidase, in terms of ferricyanide reduction, is 3:1 for bovine liver, and 1:12 for rabbit liver.

Future attempts to purify aldehyde oxidase from a bovine source, therefore, should start with a far greater initial tissue mass (probably around 600 g). Although this would make initial stages more cumbersome, by increasing the number of ammonium sulphate precipitation stages, the crude homogenate could be quickly reduced to a more manageable volume. There is no reason why the method used to purify the enzyme from rabbit liver could not be used for purification of aldehyde oxidase from bovine or, indeed, human liver.

4.10 Recommendations for future work

4.10.1 Generation of polyclonal, anti-(XOR) antibodies

The presence of circulating anti-(XOR) antibodies prior to immunization, should be determined and the effect on post-immunization titres assessed. Immunization protocols should use the lowest amount of immunogen that still provokes a good response. Although this will have to be determined empirically, it may be possible to immunize effectively with less than 100 μg BXO or HXO.

When the rabbit has a relatively high pre-immunization anti-(XOR) titre, it may be prudent to sacrifice the animal 10 days after the first immunization. In any case, antiserum collected from each bleed should be tested for immune complexes, and batches of antiserum should not be pooled. If immune complexes are present, care should be taken to remove the contaminating enzyme during IgG preparation, either by precipitation or gel filtration.

4.10.2 Production of monoclonal anti-(XOR) IgG

Investigation of the effect of pre-immunization anti-(XOR) titres on successful, IgG-secreting hybridoma production should be pursued. Tail bleeds from a population of BALB/c mice should be screened for anti-(XOR) antibody. Anti-(XOR)-positive mice should be immunized and splenocytes used for hybridoma production, in tandem with splenocytes from immunized anti-(XOR)-negative mice.

As discussed previously, fusions using splenocytes from other strains of mouse or, more radically, other species of laboratory animal, may present alternatives to the currently unsuccessful in-house method of anti-(XOR) IgG production.

If mice with pre-immunization anti-(XOR) titres are not found, and other strains or species are not available, immunizations schedules should be performed over the maximum length of time allowed under Home Office Regulations, using the minimum amount of XOR (~ 10 μg).

As demonstrated by the monoclonal H2C2, it is important to establish the source of the mAb's epitope before starting any immunological investigations. Western blots should normally allow visualization of the target protein but, in some cases, the conformational change induced by the SDS-PAGE and subsequent Western blotting procedure may disrupt the epitope. Although coupling of the antibody to CNBr-inactivated Sepharose 4B is a relatively time-consuming procedure, it provides reliable indication of the antigen. If the nature of the antigen is suspected (e.g. the 80 kDa contaminant), and a homogeneous preparation of the suspected protein is available, mAb/suspected antigen interaction can be estimated by ELISA or, more rapidly, using plasmon surface resonance measurements (Appendix III).

Once IgG-secreting hybridoma lines have been established, it will be necessary to determine the molecular weights of the light and heavy chains (from SDS-PAGE gels). It would also be useful to record weight per unit volume (this measurement is more useful than titre in routine mAb work), and the affinity of each antibody population (derived from Scatchard plot analysis).

4.10.3 Replacing pAb with mAb

As monoclonal anti-(XOR) IgG was not available during this project, various experiments have been described using polyclonal IgG, with the knowledge that such heterogeneous systems are not ideal. When one or more hybridomas secreting high-affinity anti-(XOR) monoclonal IgG becomes established, the monoclonal IgG can be substituted for polyclonal IgG in the methods described, increasing specificity and reproducibility.

Again, once hybridoma supernatant is routinely available, a standard purification strategy can be established. In order to obtain mAb preparations of high purity, it will be necessary to determine:

- pI
- pH stability
- ionic stability at high and low ionic strengths
- hydrophobicity.

For preparations above 50% purity, a standard protocol will incorporate clarification stages, protein A affinity purification, ion exchange chromatography (the type depends on the pI), followed by gel filtration.

Purified anti-(XOR) mAb can be substituted directly for pAb on affinity columns. The protocol for cross-linking antibody to protein A will be similar. Similarly, anti-(XOR) mAb/protein A/Sepharose 4B-beads prepared in the same way could be added directly to small volumes of tissue extract, and used to extract antigen.

High-affinity IgG will also be a powerful immunohistochemical tool. Although Hellsten-Westing (1993) has reported immunohistochemical investigation with an anti-(XOR) mAb in human tissues, such antibodies will be of great value in cell culture work and, in particular, investigation of molecular activation–deactivation mechanisms.

The nature of high-affinity anti-(XOR) IgG association with aldehyde oxidase will be of considerable interest. The preparation of purified bovine and human aldehyde oxidase (suggested modifications to the protocol for rabbit aldehyde oxidase have been made previously; Discussion 4.8) will allow more detailed comparisons and cross-reactivity studies to be pursued. Specific anti-(aldehyde oxidase) mAb would complement anti-(XOR) studies. It is interesting that all immunological investigations of XOR in mammalian tissues have ignored the effects of cross-reactive aldehyde oxidase.

One of the most important questions raised by this project concerns the preliminary indication that an inhibitor protein may be present *in vivo*, in non-cardiac tissues. Further investigation with a range of anti-(XOR) mAbs will determine whether this is, in fact, an XOR fragment.

Isolation and characterization of the protein corresponding to the 64-kDa protein will be necessary to study its interactions with XOR in detail.

With increasing knowledge of the primary, secondary and tertiary structure of XOR (see, for example, Appendix IV) and increasingly powerful molecular engineering techniques, it should be possible to make specific antigens comprising specific parts of the XOR enzyme. For example, by large-scale production of enzyme fragments in their proposed altered conformational states, it may be possible to generate highly specific mAbs specific for different conformational states. These would then allow thorough investigation of the nature of the enzyme, under physiological and pathophysiological conditions. In addition, specific anti-(BXO) and anti-(HXO) mAbs will allow investigation of the origin of naturally occurring anti-(XOR) antibodies.

Xanthine oxidoreductase is a complex enzyme that can exist in different states *in vitro*. Because of its potential to generate free radicals, its distribution and activity has been studied extensively, but reported results have often been disparate.

Biochemical studies of the enzyme cannot encompass the effects of different enzyme forms. These studies should, therefore, be complemented with immunological investigations of XOR protein. The ideal system will incorporate a range of highly specific anti-(XOR) monoclonal antibodies, class IgG. Although the production of these antibodies was unsuccessful during this project, the experience gained will be of great assistance to the next investigator. Similarly, the in-house methodology established for the use of IgG in the study of XOR will be easily modified to incorporate anti-(XOR) mAbs.

Preliminary experiments with anti-(XOR) pAb indicate that an 'HXO-like' protein is present in liver, heart and smooth muscle. The low specific activity of the heart enzyme is in agreement with reports indicating that most of the heart enzyme is in a form largely inactive in conventional assays of XOR activity. This is the first report of XOR activity in human heart that has been deep frozen. Very early results also suggest the existence of an XOR inhibitor in liver but not in heart.

Appendix I

Names and addresses of suppliers

Amersham International plc
Lincoln Place
Green End
Aylesbury
Bucks

BDH Chemicals Ltd
Chadwell Heath
Essex

Becton Dickinson UK Ltd
Between Towns Road
Cowley
Oxford

Bibby Sterilin Ltd
Tilling Drive
Stone
Staffs

Bio-Rad Laboratories Ltd
Marylands Avenue
Hemel Hempstead
Herts

Biozyme Laboratories Ltd
Unit 6
Gilchrest Thomas Estate
Gwent
S Wales

Gelman Sciences Ltd
Harrowden Road
Brackmills
Northampton

Gibco BRL
(Life Technologies Ltd)
Trident House
Paisley
Scotland

ICN Biomedicals Ltd
Thame Park Business Centre
Wenman Road
Thame
Oxon

Imperial Laboratories Ltd
West Portway
Andover
Hants

Labsystems
Unit 5
Ringway Centre
Edison Road
Basingstoke
Hants

Millipore (UK) Ltd
11-15 Peterborough Road
Harrow
Mddsx

National Diagnostics
Unit 4
Fleet Business Park
Itlings Lane
Hessle
Hull

Pharmacia Biotech Ltd
23 Grovesnor Road
St Albans
Herts

Pierce
(Pierce and Warriner UK Ltd)
Upper Northgate Street
Chester

Sigma Chemical Co. Ltd
Fancy Road
Poole
Dorset

Vector Laboratories Ltd
16 Wulfric Square
Bretton
Peterborough

Whatman Ltd
Twyfords
Reading
Berks

Appendix II

Improving the in-house purification of HXO from human milk was an ongoing project in our laboratory. As HXO at various stages of the preparation is referred to in the text, an outline of the standard protocols used is presented.

Preparation of crude extract from human milk

To a sample of fresh (i.e. expressed within 8 hours) human milk, were added:

EDTA	1 mM
DTT	2.5 mM
Sodium salicylate	1.25 mM
Leupeptin	1 μg/ml
Antipain	1 μg/ml
Aprotinin	1 μg/ml
Pepstatin A	1 μg/ml.

The milk was stirred, then centrifuged at 3000 *g*, 6°C, for 30 minutes. The cream was collected using a spatula, and resuspended in an equal volume 0.2 M dipotassium hydrogen phosphate buffer, containing 1 mM EDTA, 1.25 mM sodium salicylate and 2.5 mM DTT. The mixture was stirred for 1 hour at 4°C, and then centrifuged at 3000 *g*, 6°C, for 30 minutes. The aqueous supernatant was collected and cold (4°C) 15% (v/v) butan-1-ol was added, with constant stirring. Ammonium sulphate, 15% (w/v), was added with constant stirring, and the stirring was continued for 1 hour. The mixture was centrifuged at 13,000 *g*, 4°C, for 20 minutes. Ammonium sulphate, 20% (w/v) was added to the supernatant and, again, the mixture was stirred for 1 hour. The mixture was

centrifuged at 10,000 *g*, 4°C, for 30 minutes; the precipitate was resuspended in an appropriate buffer (depending on the next stage). The resuspended pellet was dialysed against the same buffer overnight.

Calcium phosphate chromatography

The crude HXO preparation was resuspended in, and dialysed against, 200 mM disodium hydrogen phosphate buffer, pH 6.0, containing 1 mM EDTA, 1.25 mM sodium salicylate and 2.5 mM DTT. A calcium phosphate chromatography column was equilibrated with the same buffer. The crude HXO preparation was slowly applied to the column. Unadsorbed protein was washed from the gel with the starting buffer. Bound HXO was eluted using the same buffer containing 5% (w/v) ammonium sulphate.

Although this method did, on occasions, yield pure HXO (as judged by SDS-PAGE and A_{450}:A_{280} ratio close to 5) it was not reproducible and the calcium phosphate chromatography columns were very short-lived. Also, the 80 kDa contaminant seemed to co-elute with HXO using this method, and an alternative was sought.

Heparin chromatography

This provided a good alternative to calcium phosphate chromatography. The results were reproducible and the resulting HXO preparation was routinely of a higher purity.

The crude HXO preparation was resuspended in, and dialysed against, 25 mM sodium phosphate buffer, pH 7.5, containing 1 mM EDTA, 1.25 mM

sodium salicylate and 2.5 mM DTT. It was slowly recycled through a 10 ml heparin-agarose chromatography column, that had been equilibrated with 25 mM sodium phosphate buffer, pH 7.5, containing 1 mM EDTA, 1.25 mM sodium salicylate and 2.5 mM DTT. Bound protein was eluted with the same buffer containing a range of sodium chloride concentrations, 0.1–1 M. Bound HXO eluted with buffer containing 0.4 M sodium chloride.

Further purification

Further attempts to purify the post-heparin HXO used gel filtration, hydrophobic interaction, or folate affinity chromatography. None of these, however, became routine during the course of the project.

Appendix III

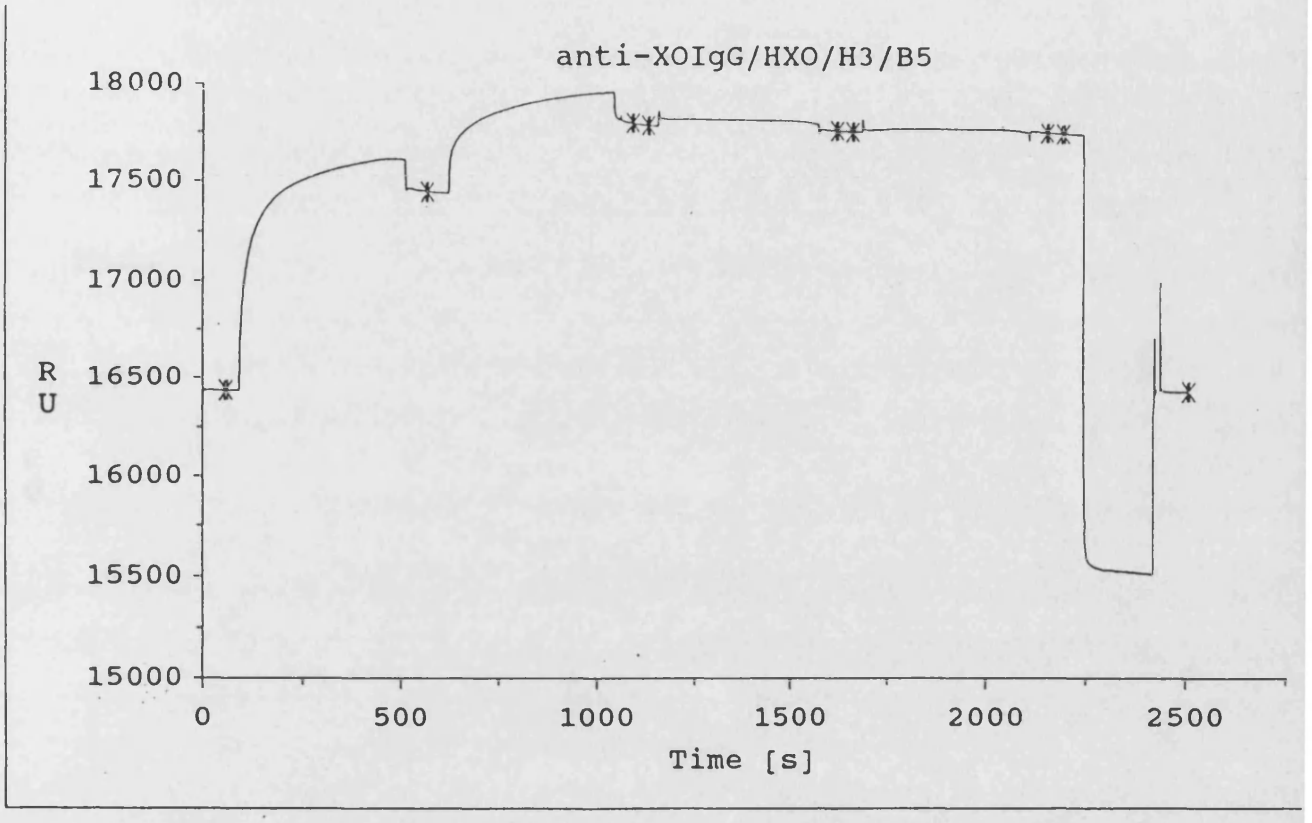
BIAcore surface plasmon resonance measurement of HXO/mAb interaction

Samples of monoclonal antibodies H2C2 (anti-XO IgG on sensorgrams), B5B4 (B5 on sensorgrams), and H3G7 (H3 on sensorgrams) were sent to The London Hospital for analysis.

The BIAcore surface plasmon resonance sensor measures antibody/antigen association in real time. Measurements can, therefore, be used as an indicator of antibody affinity.

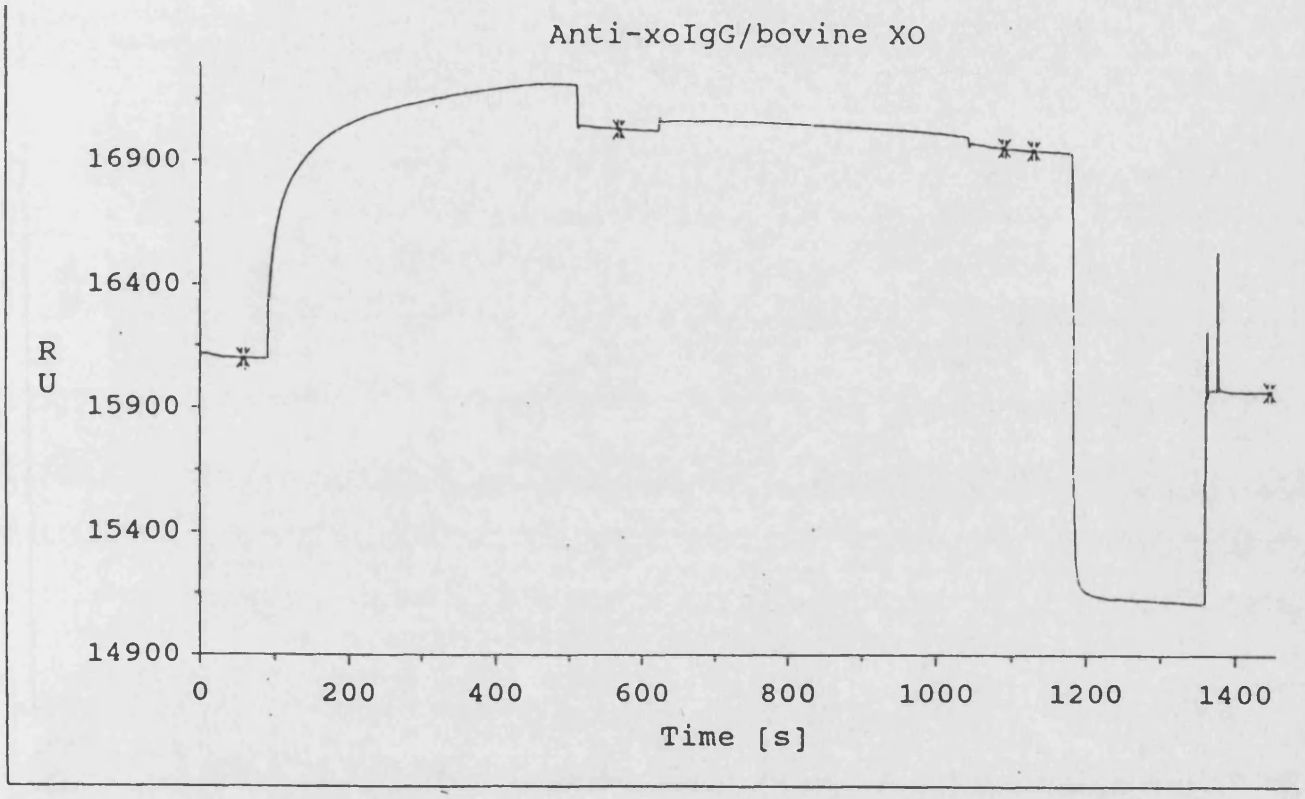
As the HXO used was from a calcium phosphate preparation (Appendix II), it was likely that it ~~was~~ contained the 80 kDa contaminate. If this was the case, then Figure 25 can be interpreted as follows. H2C2 binds the 80 kDa contaminant in the HXO preparation. Subsequent addition of H3G7 and then B5B4, however, causes no increase in RU, as neither antibody recognises the immobilized protein. Similarly, Figure 26 clearly demonstrates a lack of recognition by H2C2 for BXO. When H3G7 or B5B4 were used as the primary antibody, however, H2C2 was still able to interact with an immobilized antigen (Figures 27 and 28, respectively).

Taken together, these results imply that the 80 kDa and HXO proteins are associated in the HXO preparation. On binding H2C2, the epitope(s) for both H3G7 and B5B4 are obstructed and, hence, the relatively bulky IgM antibodies are not able to bind. If the HXO binds to the IgM antibodies first, however, the IgG antibody is till able to interact with its antigen. Figures 27 and 28 also indicate that H3G7 and B5B4 have relatively low affinity for HXO.



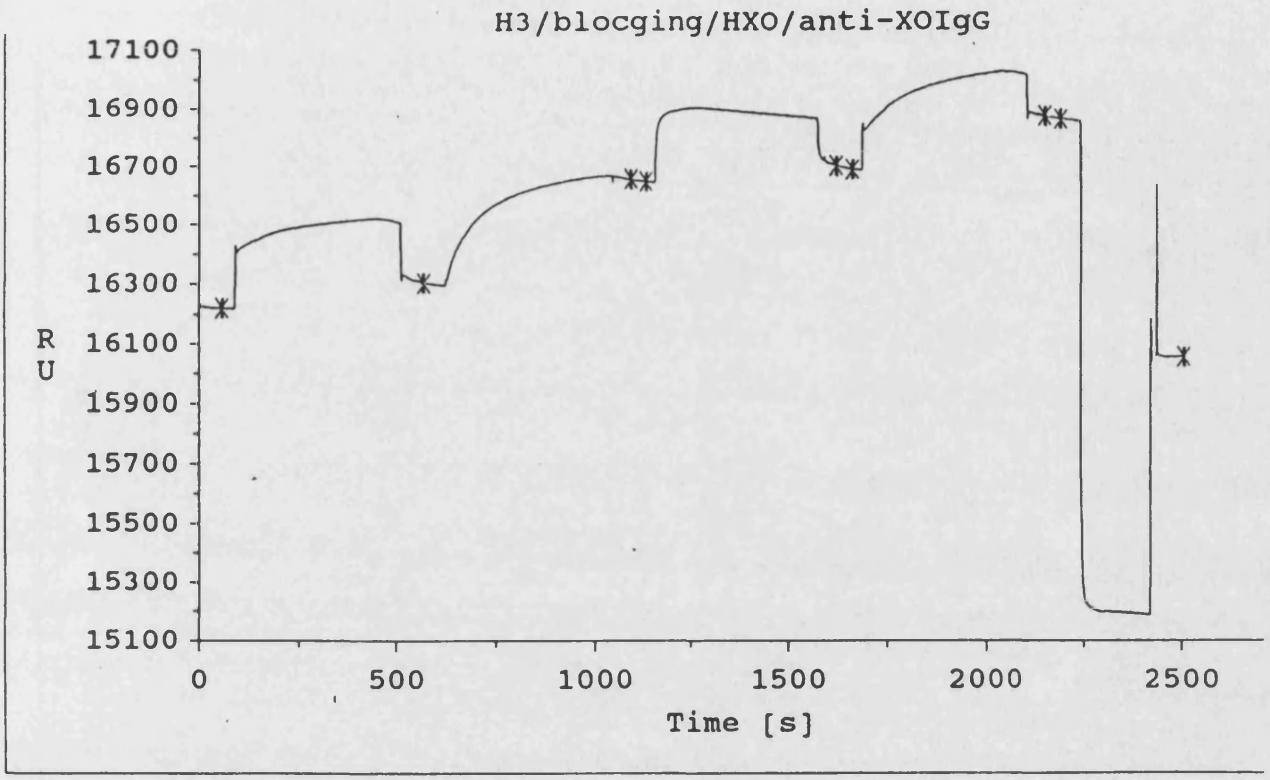
APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
XO	2	57.50	5.00	16434	0.59042	-0.18237	0.53874	YES	0	baseline
XO	2	567.40	5.00	17454	1.74004	-0.05346	1.94221	NO	1020	anti-XOIgG
XO	2	1088.70	5.00	17803	1.94032	-0.94079	0.91312	NO	1369	HXO
XO	2	1128.70	5.00	17791	1.27783	-0.58901	0.72335	NO	1357	r2
XO	2	1619.80	5.00	17763	1.16365	-0.58008	0.46952	NO	1329	H3
XO	2	1659.80	5.00	17758	0.98452	-0.23242	0.98755	NO	1324	r2
XO	2	2150.50	5.00	17758	1.39351	-0.29224	1.43306	NO	1324	B5
XO	2	2190.50	5.00	17752	0.30583	0.08309	0.29446	NO	1318	r2
XO	2	2507.40	5.00	16438	0.69305	-0.04833	0.76823	NO	4	reg

Figure 25. BIAcore surface plasmon resonance trace showing relative affinity of H2C2 (anti-XOIgG in key) for 'HXO' (i.e. possibly the 80 kDa contaminant of early HXO preparations), and the subsequent lack of recognition by H3G7 (H3 in key) and B5B4 (B5 in key). RU = response, which is a relative value corresponding to association and dissociation between the test proteins.



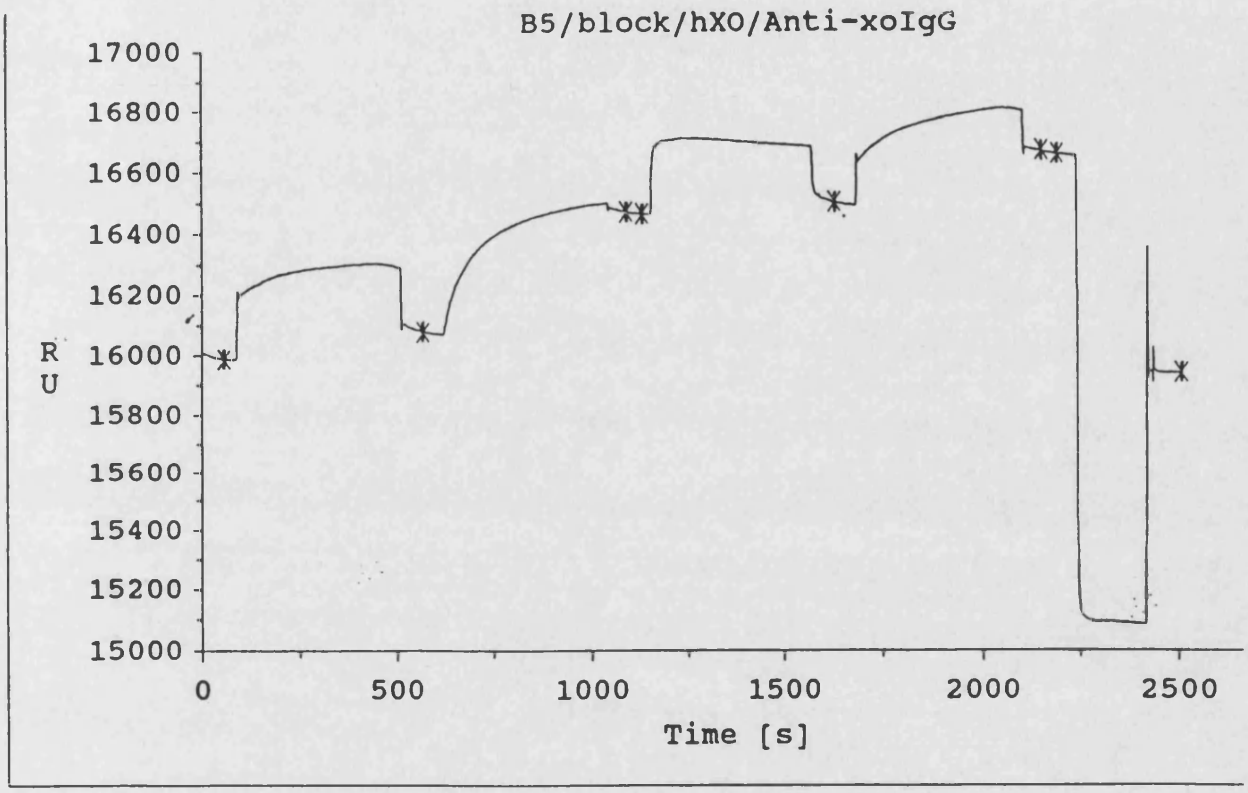
APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
XO	2	57.60	5.00	16098	1.01242	0.16220	1.02883	YES	0	baseline
XO	2	567.50	5.00	17034	0.90501	-0.35028	0.73153	NO	936	anti-xoIgG
XO	2	1088.80	5.00	16959	1.81778	-0.66183	1.52745	NO	860	boviXO
XO	2	1128.80	5.00	16949	1.08552	0.12074	1.12461	NO	851	r2
XO	2	1445.80	5.00	15975	0.87820	-0.34368	0.70429	NO	-123	reg

Figure 26. BIAcore surface plasmon resonance trace showing lack of affinity of H2C2 (anti-XOIgG in key) for BXO (boviXO in key). RU = response, which is a relative value corresponding to association and dissociation between the test proteins.



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
XO	2	57.60	5.00	16220	0.45379	0.00399	0.50729	YES	0	baseline
XO	2	567.50	5.00	16305	0.58865	-0.28666	0.27132	NO	85	H3
XO	2	1088.60	5.00	16658	1.83053	-0.85279	1.00342	NO	439	blocking
XO	2	1128.60	5.00	16651	0.44684	-0.08583	0.46622	NO	432	r2
XO	2	1619.60	5.00	16704	2.43425	-1.02204	1.68433	NO	484	HXO
XO	2	1659.60	5.00	16692	0.33968	-0.01959	0.37756	NO	472	r2
XO	2	2150.40	5.00	16877	1.64261	-0.61853	1.30344	NO	657	anti-xoIgG
XO	2	2190.40	5.00	16867	1.04186	-0.29794	0.98412	NO	647	r2
XO	2	2507.00	5.00	16060	1.43575	0.02313	1.60449	NO	-160	reg

Figure 27. BIAcore surface plasmon resonance trace showing relative affinity of H3G7 (H3 in key) for HXO, and subsequent association of H2C2 (anti-XOIgG in key). RU = response, which is a relative value corresponding to association and dissociation between the test proteins.



APROG	FC	Time	Window	AbsResp	SD	Slope	LRSD	Baseline	RelResp	Id
XO	2	57.40	5.00	15988	0.85962	-0.31401	0.70164	YES	0	baseline
XO	2	567.30	5.00	16079	1.02495	0.12084	1.11771	NO	91	B5
XO	2	1088.20	5.00	16471	1.96847	-0.90234	1.13198	NO	483	Blocking
XO	2	1128.20	5.00	16463	0.77676	-0.19235	0.76962	NO	476	r2
XO	2	1628.90	5.00	16500	1.08143	-0.24029	1.09966	NO	512	HXO
XO	2	2149.90	5.00	16668	1.54146	-0.62483	1.12342	NO	680	anti-XOIgG
XO	2	2189.90	5.00	16658	0.58971	0.22349	0.46495	NO	670	r2
XO	2	2506.70	5.00	15944	0.59568	0.14919	0.58836	NO	-44	reg

Figure 28. BIAcore surface plasmon resonance trace showing relative affinity of B5B4 (B5 in key) for HXO, and subsequent association of H2C2 (anti-XOIgG in key). RU = response, which is a relative value corresponding to association and dissociation between the test proteins.

Appendix IV

In order to demonstrate that HXO was immunogenic, and problems generating anti-(XOR) mAbs were not caused by restricted epitopes, the HXO sequence was analysed using the program PeptideStructure. This is part of the Genetics Computer Group suite of programs (Devereux *et al.*, 1984). Figure 29 shows the antigenicity profile of the protein (i.e. points in secondary structure where antigenic index > 1.2). Antigenic index (AI) is calculated by summing several weighted measures of the protein's secondary structure; it is a measure of the probability that a region is antigenic.

This method of AI determination was first described by Jameson and Wolf (1988), and uses a formula which incorporates measurement of hydrophilicity, surface probability, flexibility, and Chou-Fasman and Robson-Garnier predictions of secondary structure.

From Figure 29, it appears that particularly antigenic regions lie between residues 150 and 200, and 800 and 1150.

Figure 30 shows plots for hydrophilicity, surface probability, flexibility, AI, Chou-Fasman and Robson-Garnier predictions of secondary structure, and predicted glycosylation sites on HXO.

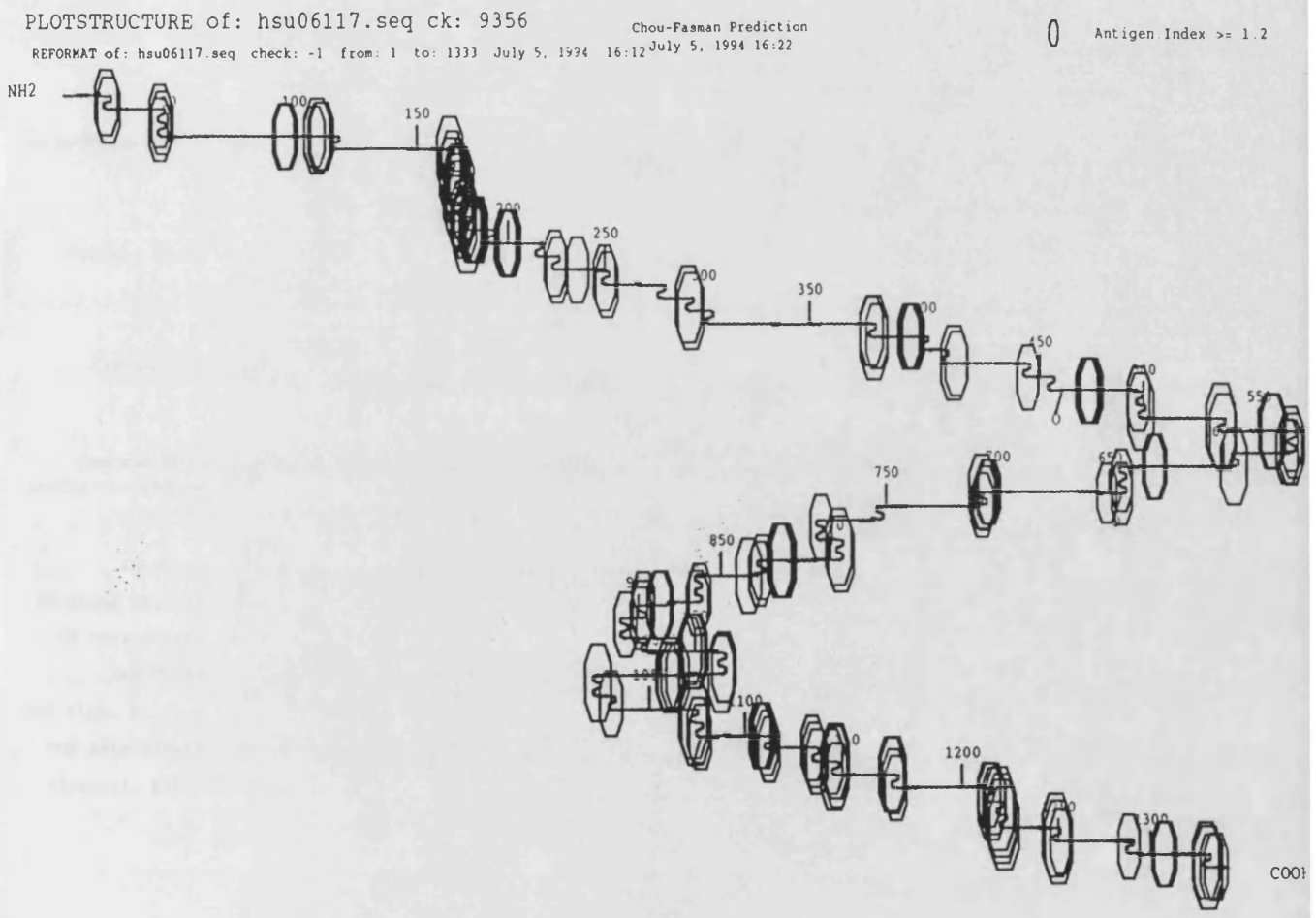


Figure 29. Antigenicity profile of HXO, determined using part of the Genetics Computer Group suite of programs (Devereux *et al.*, 1984).

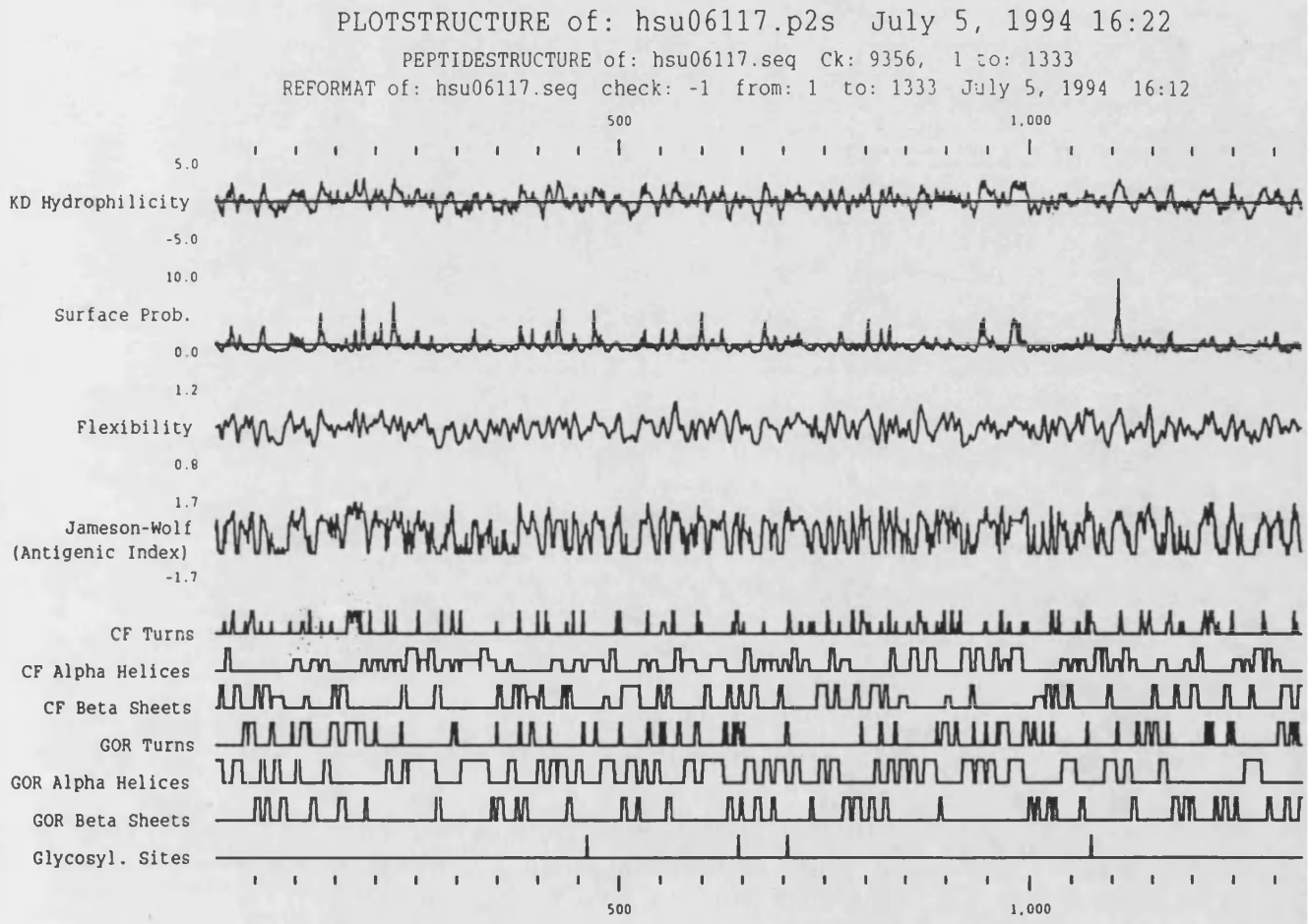


Figure 30. Summary of secondary structure characteristics of HXO, determined using part of the Genetics Computer Group suite of programs (Devereux *et al.*, 1984).

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